

CHARACTERIZATION OF THE ADP-RIBOSYLATED
PROTEOME BY MASS SPECTROMETRY

by
Casey M. Daniels

A dissertation submitted to Johns Hopkins University in conformity
with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
July 2015

© 2015 Casey M. Daniels
All Rights Reserved

Abstract

ADP-ribosylation is a post-translational modification synthesized by poly(ADP-ribose) polymerases (PARPs) and is the target of the chemotherapeutic PARP inhibitors. This protein modification has proven clinically important in DNA damage repair, inflammatory processes and cell death, and biologically important in nearly all cellular processes. Research into ADP-ribosylation has been limited, however, by a lack of mass spectrometry (MS) based methods for identifying protein modification sites. My thesis work has provided a robust, unbiased method for identifying ADP-ribosylation sites by simplifying this otherwise complex modification down to its phosphoribose attachment site, a 212 Dalton molecule which is easily identifiable by MS and can be enriched using standard phosphoenrichment matrices. Through the development and validation of this assay we (1) identified the automodification sites on PARP-1, a critical protein for DNA repair, among other cellular processes, (2) identified the ADP-ribosylation sites on endogenous proteins, (3) showed that the macrodomain containing protein MacroD2 has ADPr hydrolase activity, and (4) found that Nudix enzymes, otherwise known for degrading free ADPr into phosphoribose and AMP, are capable of degrading protein-conjugated poly- and mono(ADP-ribose). This thesis also contains a chapter dedicated to benchmarking the current state of the ADP-ribosylation field with regard to MS based proteomics, and considerations for the future promise and potential held by MS based proteomics for the study of ADP-ribosylation.

Advisor: Dr. Anthony Leung

Thesis Readers: Dr. Cynthia Wolberger, Dr. Anthony Leung, Dr. Michael Matunis,
Dr. Rhoel Dinglasan, Dr. David Goodlett

Acknowledgements

First and foremost, I would like to thank my advisor, Anthony, for giving me the opportunity to join him in these formative years of his career – the earliest days of his independent research group. Over these last four years Anthony has provided the resources, guidance and support needed to set me on an academic research path, including supporting my participation in some of the world’s best training for mass spectrometry based proteomics – that offered by Cold Spring Harbor Laboratory and by the Max Planck Institute/Mann Lab. Other day-to-day gifts have included his generous open-door policy, his continuous efforts to fund and supply the lab, and his willingness to try new ideas, make new connections, and build on the ideas set forth by other members of his group. Finally, his proposal – that of developing an SVP-based pipeline for identifying protein ADP-ribosylation sites – formed the foundation of my thesis work, and proved both creative and elegant.

My sincerest gratitude also extends to Shao-En, who welcomed me into his research group as one of his earliest trainees. The months spent in his laboratory, and working side-by-side with him at his instrument, provided the basic training needed to pursue a career in the fields of mass spectrometry and proteomics. His support has continued unabated throughout these last four years, including a number of valuable recommendation letters, thoughtful manuscript critiques, and career guidance. Insights from our conversations have shaped the way that I think about method development and the technical challenges we seek to overcome in our pursuit of relevant, impactful proteomic research.

Mike, who has served on all of my committees, including my preliminary oral exam committee, my qualifying exam committee, and the entire duration of my thesis committee, Cynthia, who has been a wonderful committee chair and source of direction and support, and Rhoel and Dave, who have been a source of technical and career-related advice at my committee meetings from the perspective of successful mass spectrometry experts; all have provided guidance, support and

insight over many years, many meetings, and many emails. I am hugely grateful for their commitment to my training and professional, research and personal goals.

The biochemistry and molecular biology department is full of people to thank, particularly: Pierre, who provided counsel and support during my earliest days in this department, Brian, who taught me the tricks and techniques for expressing, purifying, and characterizing proteins, and Sharon, Shannon and Brandon who ensured I had the particulars of this process in order for a successful and timely graduation. I have enjoyed five years of department retreats, picnics, Christmas parties, joint lab meetings, and – perhaps most valuable – lunch room conversations. For these things I am grateful to have been a part of this department.

Finally, I would not have been able to push myself through the growing experiences and challenges offered by this department had I not had the support of my friends and family. Dave -- my loving partner and best friend -- my parents, my sister Erika, my brother Matt, and my dearest friends (namely, Michelle, Yen and Lauren and fellow lab-mates Yoshi and Christina) have been in this with me from the beginning, listening and supporting. Thank you.

Table of Contents

Abstract	ii
Acknowledgements.....	iii
Table of Contents	v
Table of Tables.....	vii
Table of Figures	vii
Abbreviations and Nomenclature	x
1 Introduction.....	1
1.1 PARPs and ADP-ribosylation: clinically and biologically important	1
1.1.1 PARPs and DNA repair.....	2
1.1.2 PARPs and transcription	4
1.1.3 PARPs and Mitosis	6
1.1.4 PARPs in RNA regulation.....	8
1.1.5 PARPs and cell death	9
1.1.6 PARPs and inflammatory signaling.....	11
1.1.7 The therapeutic inhibition of PARPs	12
1.2 Mass spectrometry based proteomics for the study of post-translational modification networks.....	12
1.3 Applying mass spectrometry based proteomics to the study of ADP-ribosylation.....	13
1.4 Figures	15
1.5 References.....	23
2 Phosphoproteomic Approach to Characterize Protein Mono- and Poly(ADP-ribosylation) Sites from Cells.....	35
2.1 Abstract.....	36
2.2 Introduction.....	36
2.3 Materials and Methods.....	38
2.4 Results	45
2.5 Discussion.....	51
2.6 Acknowledgements	55
2.7 Figures	56
2.8 Supplementary Figures	65
2.9 Supplementary Text	71
2.10 Supplementary Spectra	73

2.11	References.....	85
3	Nudix Hydrolases degrade protein-conjugated ADP-ribose.....	89
3.1	Abstract.....	90
3.2	Introduction.....	90
3.3	Materials and Methods.....	92
3.4	Results	96
3.5	Discussion.....	99
3.6	Author Contributions	100
3.7	Acknowledgements	100
3.8	Figures	101
3.9	References.....	107
4	The Promise of Proteomics for the Study of ADP-ribosylation	110
4.1	Abstract.....	111
4.2	Introduction.....	111
4.3	Investigating the ADP-ribosylated Proteome by Mass Spectrometry: Challenges	113
4.4	A Draft of the ADP-ribosylated Interactome.....	113
4.5	Characterizing ADP-ribosylation at the Level of the Amino Acid Attachment Sites	116
4.6	Defining Target Specificity for Addition and Removal of ADP-ribosylation.....	120
4.7	Distinguishing between sites of MAR- and PARylation	122
4.8	Free/Conjugated, Branched/Linear: the many forms of Poly(ADP-ribose)	124
4.9	Assessing the physiological relevance of ADP-ribosylation sites	125
4.9.1	Site Occupancy.....	125
4.9.2	Top-down Proteomics	127
4.10	Conclusions	128
4.11	Acknowledgements	128
4.12	Figures	129
4.13	Supplementary Figures	137
4.14	Supplementary Text	139
4.15	Supplementary Tables.....	142
4.16	References.....	143
5	Conclusions and Future Directions	151
5.1	Future Development - Exploring the ADP-ribosylated proteome to a greater depth.....	151
5.2	Future Applications.....	153
5.2.1	Characterizing protein acceptors of ADP-ribose	153

5.2.2	Identifying critical changes in the ADP-ribosylated proteome	154
5.2.3	Searching for biomarkers of disease, treatment and drug resistance.	155
5.3	Final Thoughts	156
5.4	Figures	157
5.5	References	162
	Appendices	166
6	Characterizing phosphorylation on MacroD2	166
6.1	Abstract	166
6.2	Introduction	166
6.3	Materials and Methods	167
6.4	Results	168
6.5	Discussion	170
6.6	Acknowledgements	171
6.7	Figures	172
6.8	Supplementary Spectra	176
6.9	References	180
	Curriculum Vitae	181

Table of Tables

Table 1-1	The PARP family	17
Table 2-1	PARP-1 automodification sites identified	61
Table 4-1	A biological toolbox of ADP-ribose binding and hydrolysis protein domains	136

Table of Figures

Figure 1-1	A typical workflow for identifying protein sequences using ‘bottom-up’ LC-MS/MS	15
Figure 1-2	The structure of Poly(ADP-ribose)	16
Figure 1-3	Synthetic lethality explains the exquisite sensitivity to PARP inhibition observed in BRCA deficient cells	18
Figure 1-4	A historical view of the development of PARP inhibitors for cancer therapy	19
Figure 1-5	The Mitotic Spindle during Metaphase	20
Figure 1-6	Parthanatos: PAR-induced cell death	21
Figure 1-7	PARP inhibitors show therapeutic promise beyond cancer	22

Figure 2-1 Visualizing phospho(ribose) tags on hPARP-1.	56
Figure 2-2 Poly(ADP-ribose) is stable in the presence of neutral phosphate buffer.....	57
Figure 2-3 IMAC and MOAC enrichment of phospho- & phospho(ribose) peptides.	58
Figure 2-4 Serial enrichment of phospho- and phospho(ribosyl)ated peptides out of a complex mixture.....	59
Figure 2-5 Proximal phospho(ribosyl)ation sites.	60
Figure 2-6 Phospho(ribosyl)ation on peptide terminal lysine.....	62
Figure 2-7 Endogenous ADP-ribosylation of Arginine.	63
Figure 2-8 The effect of macrodomain enrichment on the (ADP-ribosyl)ated amino acid profile. ...	64
Figure 3-1 Purification of snake venom phosphodiesterase for the digestion of protein-conjugated ADP-ribose.....	101
Figure 3-2 ADPrases are ineffective against protein-conjugated ADP-ribose.	102
Figure 3-3 Single domain Nudix hydrolases show activity against protein-conjugated PAR.....	104
Figure 3-4 Human Nud16 degrades protein conjugated ADPr.	105
Figure 3-5 RppH and Nud16 for the generation of phosphoribose as an ADPr tag for mass spectrometry.	106
Figure 4-1 The PARP family	129
Figure 4-2 Processes enriched in the ADP-ribosylated interactome	130
Figure 4-3 ADP-ribosylation attachment sites	131
Figure 4-4 ADP-ribosylation tags	132
Figure 4-5 PARP Substrate Specificity	134
Figure 4-6 PARP-1 automodification sites.....	135
Figure 5-1 Pipeline for identifying ADP-ribosylation sites.	157
Figure 5-2 Snapshots showing protein ADP-ribosylation in cellular pathways.	158
Figure 5-3 A historical perspective on research into ADP-ribosylation of HNRNPA1	159
Figure 5-4 Combination therapies maximize PARPi potential as a chemotherapeutic agent.	160
Figure 6-1 MacroD2 mutations modulate ADPr binding and hydrolysis.....	172
Figure 6-2 Acquiring the interaction network of MacroD2 wildtype and mutant proteins.	172
Figure 6-3 Binding and hydrolase deficient mutants of MacroD2 are more highly phosphorylated than wildtype.....	173
Figure 6-4 Casein Kinase 1 α may be responsible for the increased phosphorylation of the mutant MacroD2 proteins.	175
 Supplementary Figure 2-1 Enriching phospho- and phospho(ribose) peptides from a complex background.....	 65
Supplementary Figure 2-2 Co-enriching phospho(ribosyl)ated peptides by IMAC and MOAC.....	66
Supplementary Figure 2-3 PARP-1 is inactivated upon exposure to 8M urea.	67
Supplementary Figure 2-4 Multiple PAR sites can exist on the same peptide.	68
Supplementary Figure 2-5 Neutral loss sequence from a doubly-modified hPARP-1 peptide.....	69
Supplementary Figure 2-6 K350 of HNRNPA1 is phospho(ribosyl)ated.	70

Supplementary Figure 4-1 Processes enriched in the ADP-ribosylated interactome (full table of Figure 2B).	137
Supplementary Figure 4-2 Comparing the ADP-ribosylated interactomes with the known ADP-ribosylated proteomes.	138

Abbreviations and Nomenclature

μg	microgram
μL	microliter
ADPr	ADP-ribose
ADPrase	ADP-ribose pyrophosphatase
ADP-HPD	Adenosine diphosphate (hydroxymethyl)pyrrolidinediol
AIF	Apoptosis Inducing Factor
AP	Affinity Purification
BER	Base excision repair
BRCT	BRCA-1 C-terminus
CAM	2-chloroacetamide
CID	Collision Induced Dissociation
DMEM	Dulbecco's Modified Eagle's Medium
DOC	Sodium Deoxycholate
DSB	Double stranded break
EDTA	Ethylenediaminetetraacetic acid
FA	Formic Acid
FBS	Fetal Bovine Serum
FDR	False Discover Rate
FNR	False negative rate
FPLC	Fast Protein Liquid Chromatography
GFP	Green Fluorescent Protein
HCD	Higher energy Collisional Dissociation
HPLC	High Pressure Liquid Chromatography
HR	Homologous recombination
IMAC	Immobilized Metal Affinity Chromatography

IP	Immunoprecipitation
LC	Liquid Chromatography
LysC	endoproteinase Lys-C
MAR	Mono(ADP-ribose)
mg	milligram
mL	milliliter
MNNG	N-methyl-N'-nitro-Nnitrosoguanidine
MOAC	Metal Oxide Affinity Chromatography
MS	Mass Spectrometry
NAD	Nicotinamide Adenine Dinucleotide
NER	Nucleotide excision repair
NHEJ	Non-homologous End Joining
PA	Phosphoric acid
PAGE	Polyacrylamide Gel Electrophoresis
PAR	Poly(ADP-ribose)
PARG	Poly(ADP-ribose) Glycohydrolase
PARP	Poly(ADP-ribose) Polymerase
PARPi	PARP inhibition
PBS	Phosphate Buffered Saline
pR	phospho(ribose)
PTM	Post-translational modification
SDS	Sodium Dodecyl Sulfate
SSB	Single Stranded Break
SRSF2	Serine/Arginine rich splicing factor 2
TCEP	Tris-(2-Carboxyethyl)phosphine, Hydrochloride
TFA	Trifluoroacetic Acid
UPLC	Ultra Performance Liquid Chromatography

WGR	Tryptophan, glycine, arginine-rich domain
ZF	Zinc Finger

1 Introduction

Post-translational modifications (PTMs) regulate the stability [1], localization [2] and interaction networks [3] of cellular proteins in a dynamic and reversible fashion, expanding upon the baseline number of protein effectors in the cell through the creation of protein proteoforms (variable molecular forms of a single gene product [4]). The study of PTMs has been bolstered by the advent of mass spectrometry based proteomics, wherein proteins are digested into approximately 20 amino acid long peptides which can then be identified through fragmentation and determination of the mass-to-charge ratio of these fragments (see Figure 1-1). Recent advances in both the technology for rapidly and accurately sampling increasingly complex mixtures of peptides as well as the analytical software required to process, validate, and explore these datasets have allowed mass spectrometry based proteomics to become the gold standard for global PTM research [5]. The work presented in this thesis aims to develop mass spectrometry based proteomic tools for the study of protein ADP-ribosylation.

1.1 PARPs and ADP-ribosylation: clinically and biologically important

Protein ADP-ribosylation (poly(ADP-ribosyl)ation/PARylation and mono(ADP-ribosyl)ation/MARYlation) is a protein modification synthesized by ADP-ribosyltransferases (commonly known, and hereafter referred to, as Poly(ADP-ribose) Polymerases, or PARPs) which transfer the ADP-ribose (ADPr) group from NAD⁺ (Nicotinamide Adenine Dinucleotide) onto acceptor proteins or, in the case of PAR, existent ADP-ribosylation sites (thus forming polymers) (see Figure 1-2). The PARP family is made up of 17 proteins that contain the characteristic PARP domain, of which four (PARP-1, -2, -5a and -5b) are capable of synthesizing PAR, two (PARP-9 and -13) have no known ADP-ribosylation activity, one (PARP-4) can synthesize MAR or PAR, with the remaining enzymes responsible for MARYlation (PARP-3, -6, -7, -8, -10, -11, -12, -14, -15, 16) (see [6], Table 1-1 and Figure 4-1).

1.1.1 PARPs and DNA repair

The PARP field has emerged directly from the field of DNA repair, and it is in this context that basic discovery and research ultimately yielded a new class of chemotherapeutic drugs for patients battling ovarian cancer (later), as well as a deeper understanding of cellular roles affected by this superfamily of enzymes. It began in 1963 when PARP1 was first described as a DNA-dependent polymerase: Chambon *et al* noted that nicotinamide mononucleotide (NMN, the precursor to NAD⁺) stimulated PARP1 to produce a polyA-like product in the presence of DNA [7]. This polymer was identified as poly(ADP-ribose) (and the precursor correctly identified as NAD⁺, referred to as DPN or Diphosphopyridine nucleotide) three years later [8] and in 1980 Benjamin and Gill noted that damaged DNA, not specific DNA sequences, were responsible for activating PARP1 [9]. By the early 1990s the now steadily growing field knew that the NAD⁺-dependent automodification of PARP1, along with degradation of PAR by the poly(ADP-ribose) glycohydrolase PARG, is critical for repair of DNA single-stranded as well as double-stranded breaks in human cells [10]. The physiological impact of this basic research was validated from 1995-1998 through the creation of three independent PARP1^{-/-} mouse models, all of which proved highly sensitive to DNA alkylating agents and ionizing radiation [11-13]; MEF cells derived from these mice demonstrated sensitivity to methyl methanesulfonate (MMS, an alkylating agent and carcinogen), resulting in slower cell growth and chromosomal instability [14]. Creation of these mice also led to the discovery of PARP2, as PARP1^{-/-} MEFs were found capable of producing PAR upon stimulation with the alkylating agent MNNG [15, 16]. In 2003 a PARP2 deficient mouse model also demonstrated sensitivity to DNA alkylating agents, implicating PARP2 in DNA repair, and a PARP1^{-/-}-PARP2^{-/-} mouse model was embryonic lethal, suggesting the two enzymes are otherwise compensating for each other in critical cellular processes [17]. During this same time period PARP1 was found to interact with XRCC1 in *Saccharomyces cerevisiae*, suggesting its role in the single stranded break repair pathway known as base excision repair (BER) [18], a suggestion which was confirmed in 2003 by two separate studies which showed that PARP1 is responsible for the accumulation of XRCC1 at sites of DNA damage [19, 20], at the same time that Schreiber *et al* showed that PARP2 is also involved in

BER [21]. PARP1 would not be mechanistically linked to double stranded DNA break repair until 2006, when it was shown that PARP1 binds to double stranded breaks and competes with the NHEJ initiation factor Ku to stimulate alternative NHEJ [22] (also known as microhomology-mediated end joining, MMEJ).

Acknowledgement of PARP1's critical role in BER led to the proposal that a loss or inhibition of PARP1 (and thus BER) should be synthetically lethal in cells which are missing the high fidelity double stranded break repair pathway homologous recombination (HR) (see Figure 1-3): loss of BER would lead to an accumulation of single stranded DNA breaks which would collapse into double stranded DNA breaks and be repaired by the low-fidelity pathway NHEJ (which would also be spurred on by a lack of PARP1, see previous paragraph) instead of the unavailable high-fidelity HR. An important scenario where this could occur is in cells lacking functional BRCA genes, as BRCA1 and BRCA2 are necessary for HR [23]. In 2005 two Nature papers showed the power of this synthetically lethal approach: cells carrying mutant BRCA1 or BRCA2 are highly sensitized to PARP inhibition [24, 25]. These findings propelled PARP inhibitors into the clinical spotlight and brought on a decade of intense clinical trials which resulted in the FDA approval of the PARP inhibitor olaparib for the treatment of ovarian cancer in patients carrying BRCA mutations ([26] and Figure 1-4).

A number of other PARPs have recently been linked to genome integrity: PARPs 5a and 5b play critical roles at telomeres (see section 1.1.3), PARP3 was recently shown to be recruited to laser-induced sites of DNA damage where it acts as part of the non-homologous end joining (NHEJ) pathway [27, 28], PARP9 is recruited by PARP1 to sites of double stranded DNA breaks via its PAR binding macrodomain [29], PARP10 binds to PCNA and is important for its DNA repair activity during S-phase, potentially through its MARYlation of PCNA [30], and PARP14 assists in homologous recombination [31]. PARPs and ADP-ribosylation, then, are critically important in nearly all means of repairing damaged DNA, a role which firmly ties them to cell viability and the development and treatment of cancer.

1.1.2 PARPs and transcription

The idea that PAR can directly affect transcription was first proposed by Poirier *et al* in 1982, in which they showed that PARylated polynucleosomes are more ‘relaxed’ than their unmodified counterparts, presumably due to the electrostatic repulsion that exists between negatively charged polymers of DNA and PAR [32]. That same year Zahradka and Ebisuzaki expanded this notion by showing that PARP1 and PARG coordinate their activities to shuttle PARP1 on and off of DNA, likely opening and closing chromatin as a result [33]. While the aforementioned research teams were focused on characterizing PARP1 in transcription, Slattery *et al* was working to identify a transcription factor from HeLa cells which they knew was capable of blocking ‘random’ transcription (i.e. that induced by DNA nicking as opposed to promoter-induced transcription) by RNA Pol II, their isolation and characterization of this enzyme (which they had named TFIIC) revealed it to be PARP1, leading to their hypothesis that PARP1 binds nicked DNA with higher affinity than Pol II, competing it off of the DNA and preventing noisy transcription [34]. Thus, in less than a year, PARP1 became a *bona fide* transcriptional regulator, four years before the PARP1 gene would even be cloned [35, 36]. The free PAR polymer was shown to be a potential transcription mediator on its own in 1992: Panzeter *et al* isolated the polymer and proved that PAR binds noncovalently to all of the histones in a detergent and high-salt resistant manner but the binding is easily reversed by DNA, suggesting an elegant competitive regulation between PAR and DNA in the cell [37].

The 1990s also revealed PARP1 to be both a transcription factor (of its own expression [38]), and a regulator of transcription factors, with the seminal discovery that PARP1 interacts directly with the ubiquitous transcription factor Yin Yang 1 (YY1) [39]. This regulation of transcription factors predictably extended to include the PARylation of transcription factors as a means for regulating their interaction with DNA, as was discovered for the PARylation of the TATA-binding protein and RNA Pol II, preventing their binding to DNA, potentially preventing them from binding and transcribing a damaged gene that is undergoing repair [40, 41]. In 2001 it was discovered that PARP1 is responsible for the expression of Reg protein (a gene which induces beta-

cell replication and is important in diabetes progression) through its interaction with a cis-element in the Reg gene promoter -- PARP1 automodification abolishes this interaction and thus prevents expression of Reg [42]. The extent of PARP1's regulatory power as a transcription factor is epitomized by its repression and activation of neural gene MASH1, repression when unstimulated but activation when phosphorylated and thus enzymatically active [43].

The PARylation dependent relaxation of chromatin, as proposed by Poirier *et al* in 1982 [32], was put into physiological context in 2003 when Tulin and Spradling showed that *Drosophila* PARP is required for chromosomal loosening (puffing) in response to heat shock [44], a concept which was extended by Kim *et al* who showed that PARP1's zinc fingers bind to the DNA entering and exiting nucleosomes, competing with the linker histone to variable extents depending on its auto-PARylation status. This replacement of Histone H1 allows for a slightly less condensed chromatin formation, but is still distinct from the open, transcriptionally active chromatin and is presumably subject to PAR-dependent regulation [45]. Between 2008 and 2012 Petesch and Lis showed that PARP1 is necessary for heat shock induced nucleosome loss at the Hsp70 promoter in *Drosophila melanogaster* [46], and that PARP1 activity is required in this scenario as PARP1 spreads along the DNA, removing nucleosomes and leaving PAR in its wake [47].

The last decade has revealed that knockdown of PARP2 affects the activity of transcription factors directly, including thyroid transcription factor 1 [48] and the heterodimeric RXR/PPAR γ nuclear receptor [49], and indirectly such as through the repression of the transcription factor regulatory protein Sirt1 [50]. PARP2 also affects transcription through its regulation of Topoisomerases, enzymes that assist in transcription by managing the supercoiling introduced into DNA when strands are separated. Both PARP1 and PARP2 are known to reactivate Topoisomerase I by removing it from nicked DNA [51] and inactivate Topoisomerase β [52], suggesting a complex regulatory role in chromatin condensation.

1.1.3 PARPs and Mitosis

Mitosis in eukaryotes requires proper formation of the mitotic spindle apparatus (depicted in Figure 1-5), a structure made up of chromosomes and two proteinaceous centrosomes [53]. PARPs have been localized to the telomeres and centromeres of chromosomes as well as at the centrosomes, an organelle that serves as the microtubule organizing center at the poles of the mitotic spindle.

1.1.3.1 PARPs at Telomeres

At the end of the 20th century Susan Smith *et al* identified PARP5a (tankyrase 1) as a novel member of the PARP family capable of both PARylation activity and regulation of the telomere-protecting shelterin complex [54]. Specifically, PARP5a interacts with TRF1 (one of two telomere-binding proteins in the six-protein shelterin complex) through its ankyrin repeats and then PARylates it, resulting in the release of TRF1 from telomeres. In 2002 PARP5b (tankyrase 2) was also found to PARylate and release TRF1 from telomeres [55], and two years later Dynek and Smith showed that knockdown of PARP5a results in mitotic arrest and abnormal chromosomal separation due to a failure of the telomeres to fully resolve [56]. More recently, Donigian *et al* showed that knockdown of PARP5a results in a shortening of telomeres in a dose-dependent manner, and this shortening is due to retention of Trf1 (and thus shelterin) at the telomeres, suggesting that PARP5a allows elongation of telomeres through removal of Trf1. They also show that this pathway is absent in mice due to the absence of a tankyrase binding domain in mouse Trf1 [57]. PARP1 and PARP2 are also at telomeres where they bind TRF2 (the other telomere binding protein from the shelterin complex) and PARylate it, thus preventing it from binding telomeres [58, 59]. PARPs, then, are highly responsible for the localization of shelterin to human telomeres, and ultimately the length of these critical stretches of DNA.

1.1.3.2 PARPs at Centromeres:

Centromeres are found at the attachment site of sister chromatids and present a binding site for the kinetochore, a structure which links chromosomes to the spindle assembly and mediates proper separation of genomic material during mitosis. PARP1 was first found to localize to these

centromeres in 2000 [60], shortly followed by the identification of PARP2 at the centromeres (though their localization is not fully overlapped, PARP1 is a more diffuse pattern, PARP2 is more of a double banding pattern) and the recognition that both PARP1 and PARP2 are interacting with and PARylating the constitutive centromeric components CENPA and CENPB as well as the spindle assembly checkpoint protein BUB3 [61, 62]. The idea that PARP1 and PARP2 have slightly different roles at the centromeres is supported by their respective mouse models: PARP1^{-/-} mice show apparently normal centromere function and progression through mitosis [63] while PARP2^{-/-} mice have chromosomal mis-segregation due to kinetochore defects [17]. While the role of PARP2 at centromeres has remained understudied, PARP1 has been shown to assist in the integration of HIV-1 into centromeric DNA [64] and has proven to be required in mouse oocytes for recruitment of Bub3 to the centromere [65].

1.1.3.3 PARPs at the Centrosome

The centrosome powers and regulates the segregation and alignment of chromosomes at the metaphase plate during eukaryotic cell division [53]. In 1999 it was found that both PARP5a [66] and PARP4 [67] localize to mitotic centrosomes, followed quickly by the finding that PARP3 is a core component of the centrosome (it specifically localizes to the daughter centriole) and interferes with G1/S cell cycle progression, briefly interacting with PARP1 [68]. The potential impact of these new centrosome components was revealed in 2004 when Chang *et al* showed that PAR is enriched in both *Xenopus* and vertebrate spindles and required for proper spindle assembly [69] and Dynek and Smith noticed an aberrant (fat and/or misaligned) spindle morphology following knockdown of PARP5a [56]. In 2005 both of these groups definitively showed that PARP5a is required for successful cell division and that it is responsible for PARylating the spindle-pole organizing protein NuMA [70, 71]. In 2013 Vyas *et al* showed that six PARPs localized to the centrosomes: PARP2 and PARP3 in G₀/G₁, PARP11 and PARP5b during mitosis and PARP5a and PARP8 constitutively (they did not see PARP1 and PARP4, as had been previously reported) [72]. The role of these newly identified centrosome-localized PARPs has yet to be revealed.

1.1.4 PARPs in RNA regulation

1.1.4.1 *PARylation in cytoplasmic nucleoprotein granules (stress granules)*

The existence of cytoplasmic PAR has been known since the mid-1970s when Roberts *et al* localized it to the ribosomes as well as diffusely to the rest of the cytoplasm [73]. A few years later (5 years before PARP1 cDNA would be cloned, 15 years before PARP2 would be discovered) Elkaim *et al* published their landmark paper linking cytoplasmic RNA to mRNPs (mRNA nucleoprotein particles) and correctly proposing that the PARylation activity observed in these macromolecular complexes was distinct from the nuclear PARylation activity thus observed; it was stimulated by RNase degradation of the local RNAs, as opposed to DNA [74]. The authors also identified a number of PARylated mRNP proteins and showed that addition of snake venom phosphodiesterase (and thus degradation of PAR) caused the granules to become partially acid soluble, suggesting that PAR is a structural component of these particles [74, 75]. In 2011 Leung *et al* identified these cytoplasmic granules as stress granules and showed that PAR, two isoforms of PARG, and 6 different PARPs (5a, 12, 13.1, 13.2, 14 and 15), are localized to these granules, some of these PARPs regulate microRNA activity and PARylate Ago2, Poly(A)-binding protein, TIA1, and G3BP1 [76].

1.1.4.2 *ADP-ribosylation of RNA regulatory proteins*

By the early 1980s the field knew that HNRNPs are major PAR acceptors [77] (HNRNPs A1 & A2 would be confirmed as the major acceptors of MAR and PAR in HeLa cells in 1994 [78]) and that ADP-ribosylation of eukaryotic elongation factor 2 (EF-2) by diphtheria toxin leads to loss of RNA binding by EF-2 and its detachment from polyribosomes [79]. The trend that proteins involved in the regulation of RNA are either interacting with PAR or are PARylated themselves was recently solidified by the advent of mass-spectrometry based studies which identified the ADP-ribosylated interactome [80-83] as well as the ADP-ribosylated proteome [84, 85]; all of these studies identified a statistically significant population of RNA binding proteins [86]. This observation was solidified by a 2014 meta-analysis which showed a high correlation between RNA granule proteins and proteins that are known to be PARylated [87].

1.1.4.3 PARPs regulate viral RNA

Gao *et al*'s 2002 *Science* paper revealed that the catalytically inactive (unable to ADP-ribosylate proteins) PARP13 (termed ZAP) is responsible for removal of viral mRNAs from the cytoplasm of human cells [88]. The following year it was revealed that cells expressing PARP13 are protected from infection by four different alphaviruses (positive-sense single stranded RNA viruses) [89], and soon after that, that PARP13's 2nd and 4th zinc fingers are responsible for this viral RNA binding [90]. More recently Zhu *et al* has found that PARP13 is able to inhibit HIV-1 infection by targeting its fully processed RNA for degradation [91]. Other PARPs have also been implemented in this viral resistance mechanism: PARP7 and PARP12 were found to inhibit alphavirus infection [92] and Atasheva *et al* showed that PARP7, PARP10 and PARP12 are all interferon stimulated and prevent alphavirus infection through downregulation of viral translation [93].

1.1.5 PARPs and cell death

In the simplest of terms, apoptosis is cellular death triggered internally (and is considered a normal, healthy, and controlled form) while necrosis is triggered externally and is often uncontrolled and harmful, resulting in the release of intracellular contents and the activation of inflammatory responses. Parthanatos has been established as a separate cellular death response that is uniquely dependent upon mitochondria and poly(ADP-ribose). PAR and PARPs, however, are involved in all three subtypes.

1.1.5.1 Apoptosis

Apoptosis is a controlled form of cell death that is energy intensive, therefore one of the earliest steps in this pathway is to prevent the consumption of the energetically important coenzyme NAD⁺ by PARP1 through caspase-mediated cleavage of PARP1[94], a process first observed in 1993 [95] that explained the Berger hypothesis of cell death from 1985 [96]. This cleavage event has proven to be critical for preventing necrosis, as cells expressing an uncleavable form of PARP1 have an increased rate of necrotic cell death [97-99]. PARP5a has also been strongly tied to apoptosis, originally though its interaction with and regulation of the apoptosis inducing and suppressing Mcl-1

proteins [100] and more recently through independent demonstrations that PARP5a inhibition or silencing induces apoptosis in human lung cells [101], neuroblastoma cells [102], and osteosarcoma cell lines [103].

1.1.5.2 Necrosis

The most obvious link between PARPs and necrosis is through the energy intensive synthesis of poly(ADP-ribose), a polymer made from the energetically important molecule NAD⁺. In 1983 it was noted that inhibition of PARPs prevents energy depletion in dying cells [104] and in the 1990s it was shown that PARP1 activation (through DNA damage) causes necrosis in pancreatic islet cells [105], neuronal cells [106] and fibroblasts [107], all of which can be reversed by loss or inhibition of PARP1. PARP1 also plays roles in necrosis beyond the depletion of NAD⁺ and ATP [107, 108]: PARP1 PARylates HMGB1 (high mobility group 1 protein), leading to its subsequent acetylation, detachment from chromatin, and translocation to the cytoplasm where it is released upon necrosis, triggering inflammation [109, 110]. PARP5b has also been shown to initiate caspase-independent necrosis, a phenomenon that is reversed through inhibition of PARP5 activity [111].

1.1.5.3 Parthanatos

Parthanatos, or PAR-dependent cell death, has recently been considered a form of regulated necrosis [112] as it is based on regulated molecular pathways while still producing a necrotic outcome. Specifically, it was discovered in 2002 that PARP1 activation leads to the translocation of AIF (Apoptosis Inducing Factor) from the mitochondria to the nucleus, thus initiating caspase-independent cell death, termed parthanatos [113] (see Figure 1-6). This process is specifically dependent upon the PAR polymers as a signal, as PAR polymers (especially long, complex polymers) signal for AIF release from the mitochondria [114] and AIF must bind these polymers directly for parthanatos to occur [115]. Molecular inhibition of parthanatos was discovered in 2011 when Iduna, a PAR binding protein, was found to protect against parthanatos in neural cells [116].

1.1.6 PARPs and inflammatory signaling

The important connection between PARPs and inflammatory signaling was first suggested in 1995 when a PARP inhibitor was found to suppress immune responses in murine spleen cells [117]. Today the immunomodulatory effects of PARP inhibitors are far from understood, but two major interaction networks have come into focus: the immunostimulatory effect of PARP1 through NF- κ B signaling, and the immunosuppressive effect of PARP14 through Stat6/IL-4 signaling.

1.1.6.1 *Pro-inflammatory signaling through PARP1 mediated regulation of NF- κ B*

PARP1 was found to modulate NF- κ B signaling in 1999 when Oliver *et al* noticed that PARP1^{-/-} cells are deficient in NF- κ B dependent transcriptional activation in LPS treated macrophages [118] at the same time that Hassa and Hottiger showed that PARP1 and NF- κ B interact independent of DNA binding and that this interaction was important for NF- κ B activation [119]. It wasn't until a decade later that the complexity of this regulation became clear: on the one hand, DNA damage induces PARP1 to PARylate I κ B (NEMO), an inhibitor of NF- κ B activity, leading to its ubiquitination and subsequent degradation of I κ B, thus promoting NF- κ B activity [120, 121], on the other hand, extracellular (TLR4) signaling causes PARP1 to PARylate p65 NF- κ B, regulating its export and nuclear retention following TLR4 stimulation [122]. These unique means of PARP1 mediated NF- κ B activation were validated in 2011 when Hunter *et al* showed that PARP1's catalytic activity is required for the activation of NF- κ B following ionizing radiation but not following TNF- α signaling [123]. PARP1's close relationship with NF- κ B activity in multiple settings makes it a critical player in inflammatory signaling.

1.1.6.2 *Anti-inflammatory signaling through PARP14 mediated regulation of Stat6*

In 2007 PARP14 was identified by Goenka *et al* as CoaSt6 (co-activator of Stat6), which they showed to activate the immunomodulatory transcriptional factor Stat6 [124]. This mechanism was fleshed out by Mehrotra *et al* in 2011 when they discovered that PARP14 acts a molecular switch for Stat6: when cells are quiescent PARP14 recruits HDAC2 and 3 to IL-4 responsive promoters, silencing them. Upon IL-4 stimulation PARP14 recruits Stat6 and releases HDAC2 and 3 by

MARylating them, following which HATs occupy and activate the promoters [125]. PARP14's ADP-ribosylation activity, then, serves to inhibit inflammatory signaling, in contrast to the PARP1 dependent regulation of NF- κ B. This balance must be considered in the use of family-wide PARP inhibitors for the study or treatment of inflammation.

1.1.7 The therapeutic inhibition of PARPs

The PARP family is perhaps best known as a chemotherapeutic target for PARP inhibitors, a class of drugs propelled into the spotlight 10 years ago when it was shown that inhibition of PARP activity (and thus repair of DNA single stranded breaks via base excision repair) is a powerful 'second hit' against cancer cells carrying BRCA1 or 2 (collectively BRCA) mutations (which are defective in DNA double stranded break repair by homologous recombination) [24, 25] (see Section 1.1.1, Figure 1-3 and Figure 1-4). This synthetic lethality of PARP1 and BRCA loss has proven a successful therapy for ovarian cancer patients, with the first FDA approved PARP inhibitor, olaparib, hitting the clinic this year [26]. The potential for PARP inhibitors extends further, however, as PARP plays a more complex role in DNA damage repair than just initiation of base excision repair [126]. Furthermore, PARPs are important regulators of cell death and inflammation, processes dysregulated in most disease states. For these reasons clinical trials are ongoing for PARP inhibitors in a range of cancers (ovarian [127], breast[128], pancreatic, colorectal, gastric, lung, genitourinary, cervical, uterine, brain, head and neck, Ewing's sarcoma, and melanoma are undergoing Phase II or III clinical trials [129]) as well as 'alternative' disease states, particularly neurodegenerative, neurotraumatic, and autoimmune disorders as well as following myocardial infarctions [130] (see Figure 1-7). With so much potential for real and meaningful therapeutic applications of PARP inhibitors, the study of PARP biology has been and will continue to be full speed and optimistic.

1.2 Mass spectrometry based proteomics for the study of post-translational modification networks

Network biology refers to the study of biological systems in the context of their interactions and larger molecular complexes as a means of connecting genotype and phenotype [131]. This way of

considering molecular pathways is an emerging and powerful exercise that requires large-scale, quantitative methods for studying the unique proteins (including their many post-translationally modified forms) that make up these networks. In this context there is a clear technological front-runner: mass spectrometry (MS)-based proteomics. In its most established form, MS-based proteomics analyzes proteins from the ‘bottom up’ by digesting them down to short, 10-30 amino acid long, peptides which are then ionized before being injected into a mass analyzer where their mass-to-charge (m/z) ratio is determined before their ultimate demise in a detector which measures the number of ions (peptides) associated with that freshly determined m/z [132]. Targeted trapping (selection) of restricted m/z ratios (peptides), followed by fragmentation of these peptides, allows for peptide sequencing and determination of mass aberrations due to post-translational modifications (see Figure 1-1). It is this capability which sets LC-MS/MS (liquid chromatography separation prior to tandem mass spectrometry, as just described) apart from other means for identifying sites of post-translational modifications – in a matter of seconds a single peptide can be isolated, fragmented, and (later) sequenced and identified, allowing a researcher to localize and quantify thousands of PTMs in a sample in a matter of days, if not hours [133]. When properly controlled, these global analyses can reveal changes in signaling pathways, potentially making important connections between molecular events and cellular outcomes [134]. The fields of protein phosphorylation [133], ubiquitination [135], acetylation [136] and glycosylation [137] have emerged as powerful players in cellular biology as a result of intensive, global MS-based proteomics analyses; judging from the collection of basic and clinical research presented in section 1.1 it seems likely that ADP-ribosylation may prove to be just as intriguing and critical of a protein modification following similar interrogation.

1.3 Applying mass spectrometry based proteomics to the study of ADP-ribosylation

As research in the clinic races ahead biochemists and cell biologists have strained to keep up with developing the necessary tools to assess and optimize the many PARP inhibitors and disease states being juggled in these trials. The tools, in fact, are quite limited: there is currently no antibody

to mono(ADP-ribose), leaving a large number of ADP-ribosylation events undetected by ELISAs and immunohistological screens, and the antibodies for poly(ADP-ribose) fail to distinguish between branched and linear forms, as well as length variants. Furthermore, as this thesis work was beginning there were no proven mass spectrometry based methods for identifying ADP-ribosylation sites on endogenous proteins (for a comprehensive review, see chapter 4), a handicap which has surely restricted our understanding of the PARP inhibitors being used in clinical trials today. This need formed the basis of the work presented in this thesis, in which a mass spectrometry based proteomic pipeline has been developed and applied to the study of endogenously ADP-ribosylated proteins.

1.4 Figures

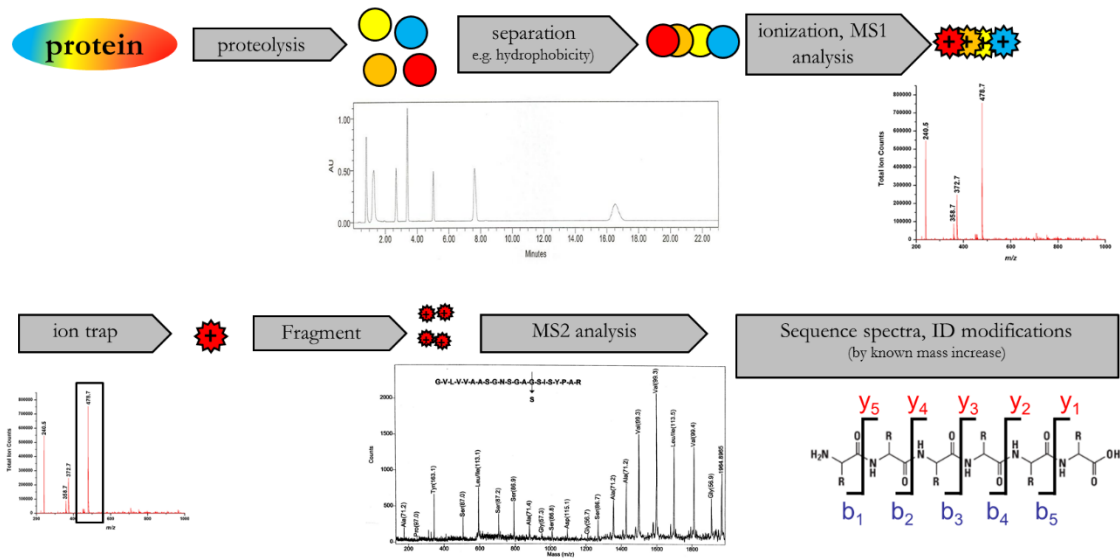


Figure 1-1 A typical workflow for identifying protein sequences using 'bottom-up' LC-MS/MS

Proteins of interest are digested to peptides through the use of proteases. These peptides are separated via liquid chromatography (LC), often based upon their hydrophobicity, and then ionized to ensure the peptides carry a charge. The mass (m), charge (z) and intensity of the peptides is determined by a mass spectrometer and displayed as intensity 'peaks' for each m/z ratio (MS1). An ion trap collects a distinct m/z ratio for fragmentation and again the m/z ratio and intensity are determined and displayed (MS2). Mass spectrometry analysis programs, along with expert validation, allows for the sequence of the peptide to be determined based on the masses of the fragmented (daughter) and intact (parent) peptide, these sequences can then be compared to protein databases to assign peptides to the proteins they are derived from. These analytical techniques also allow for the recognition of aberrant mass changes, e.g. due to a PTM.

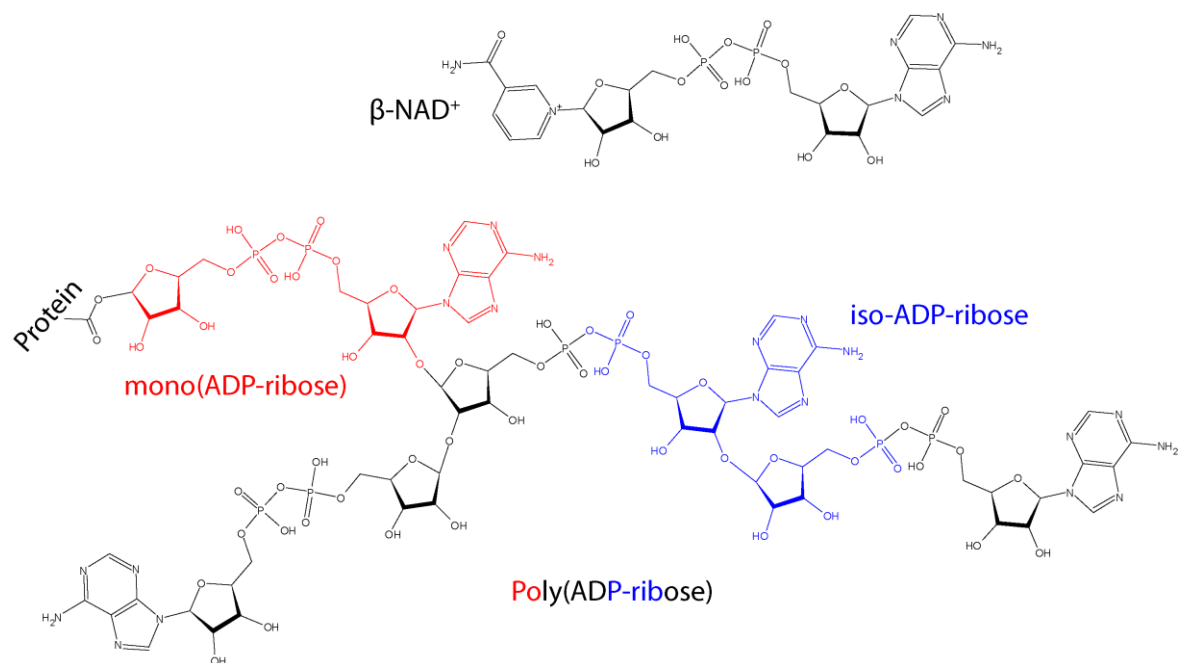


Figure 1-2 The structure of Poly(ADP-ribose)

ADP-ribose subunits are derived from β -NAD⁺ and added onto proteins in a monomeric or polymeric form. The structural subunits of PAR are depicted in red (MAR) and blue (iso-ADP-ribose).

Protein Names			Activity		
PARP	ARTD ^a /pART ^b	Aliases	-	MAR	PAR
1	1	PARP, PARS			
2	2				
3	3				
4	4	vaultPARP			
5a	5	Tnks1			
5b	6	Tnks2			
6	17				
7	14	TiPARP			
8	16				
9	9	BAL1			
10	10				
11	11				
12	12	ZC3HDC1			
13	13	ZC3HAV1, ZAP			
14	8	BAL2			
15	7	BAL3			
16	15				

Table 1-1 The PARP family

The 17 PARP family members including their current and past titles. ^a[138]^b[139].

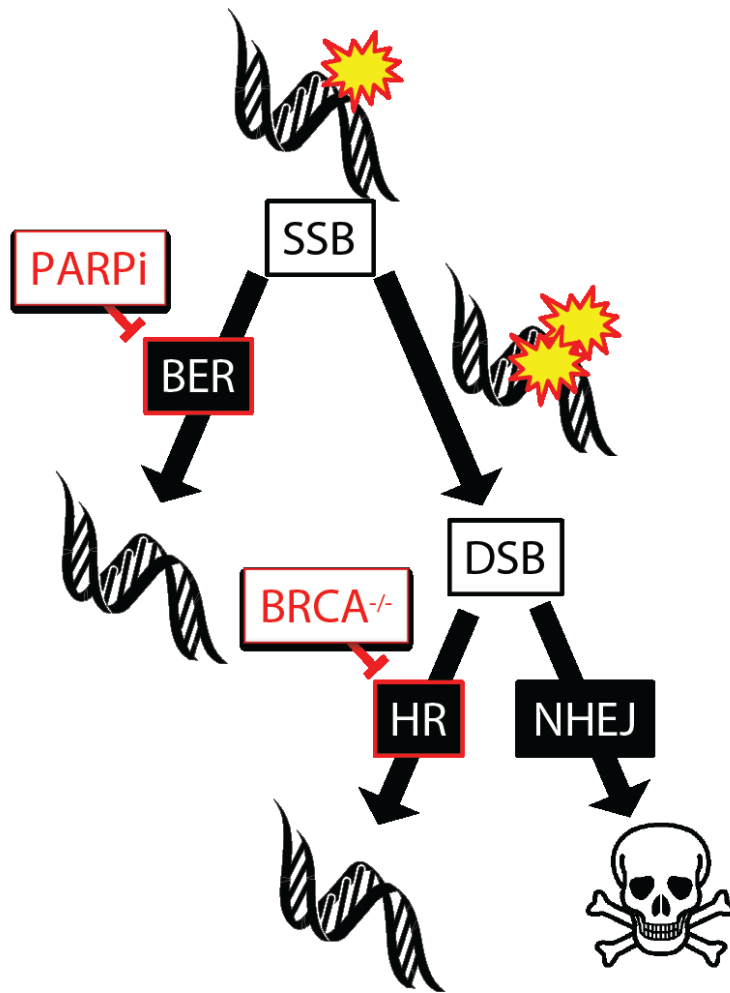


Figure 1-3 Synthetic lethality explains the exquisite sensitivity to PARP inhibition observed in BRCA deficient cells

PARP1 is involved in base excision repair (BER), a DNA repair process for single stranded breaks (SSBs); loss or inhibition of PARP1 prevents BER, thus leading to single stranded breaks deteriorating into double stranded breaks (DSBs), which are then repaired by either HR (homologous recombination) or NHEJ (non-homologous end joining). HR requires the breast cancer related proteins BRCA1 and BRCA2, if either of these proteins is mutated or missing HR cannot proceed, in which case cells are forced to rely on NHEJ, a low fidelity pathway that will ultimately lead to genomic catastrophe and death. This double-hit model is termed synthetic lethality. For more information see [128].

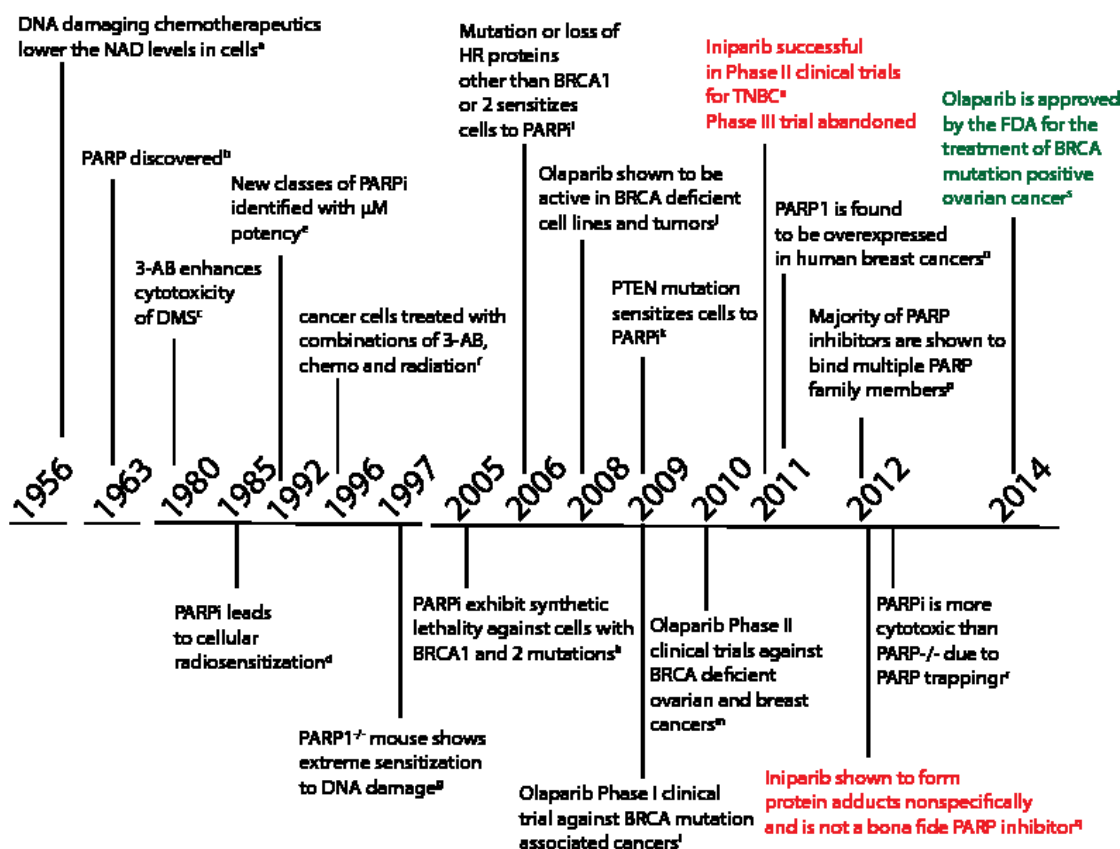


Figure 1-4 A historical view of the development of PARP inhibitors for cancer therapy.

A sustained effort including over 50+ years of research has resulted in the approval of a PARP inhibitor for the treatment of BRCA mutated ovarian cancer (green). This success was preceded by the downfall of iniparib, the most promising candidate for PARP inhibition in the clinic at the time which later turned out not to be a *bona fide* PARP inhibitor (red). 3-AB = 3-AminoBenzamide (PARP inhibitor), PTEN = Phosphatase and TENsin homolog (protein), TNBC = Triple Negative Breast Cancer, BRCA = BReast CAncer genes 1 and 2, DMS = DiMethyl Sulfide, HR = Homologous Recombination, PARPi = PARP inhibitor, FDA = Federal Drug Administration (USA).

^a[140]^b[7]^c[141]^d[142]^e[143]^f[144]^g[12]^h[24, 25]ⁱ[145]^j[146, 147]^k[148]^l[149]^m[150, 151]ⁿ[152]^o[153]^p[154]^q[155]^r[156]^s[26]

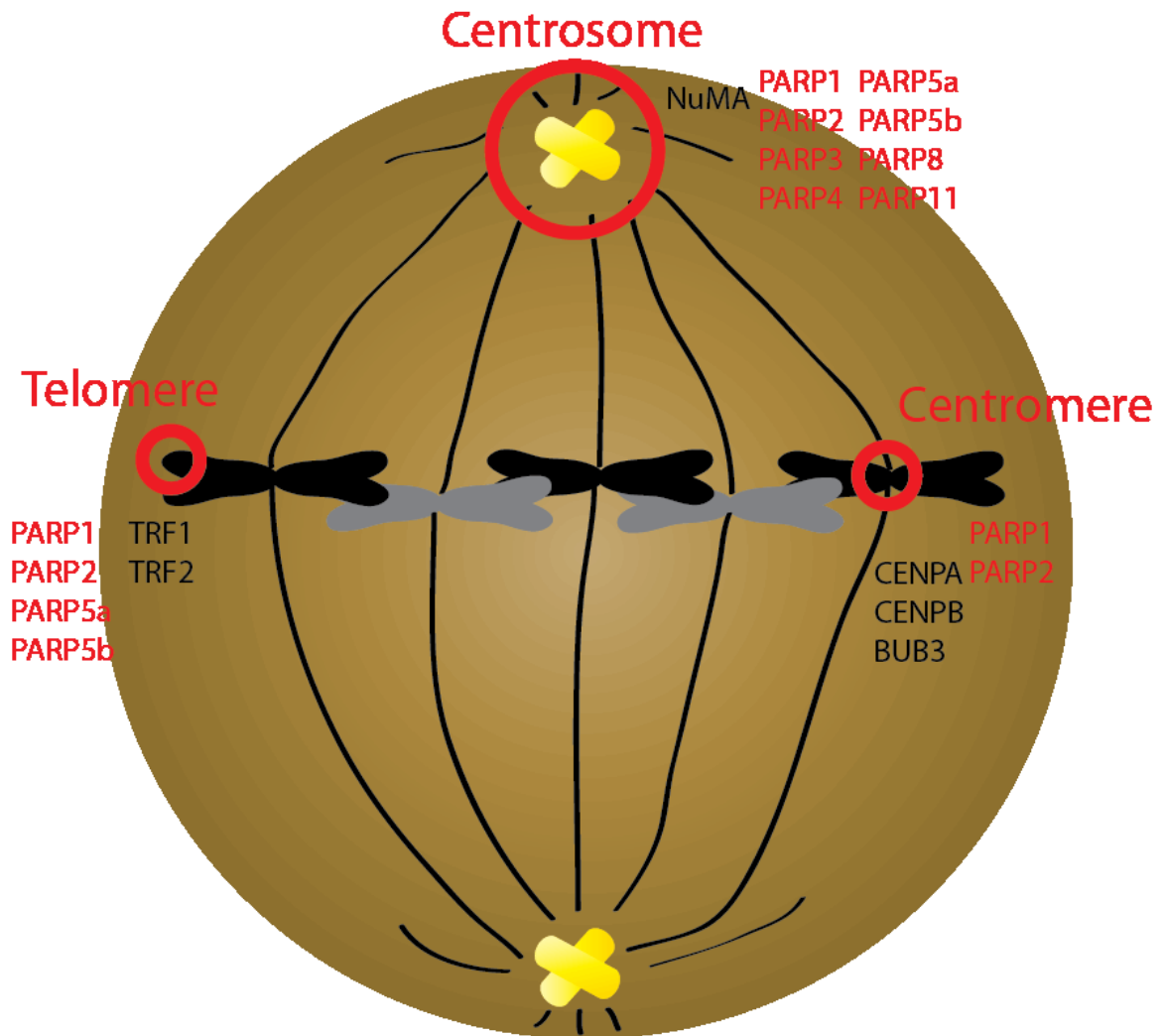


Figure 1-5 The Mitotic Spindle during Metaphase

During metaphase, the mitotic spindle is responsible for aligning the chromosomes up along the metaphase plate, as shown. PARPs are involved in this process at the level of telomere and centromere resolution (during anaphase), as well as organization of the centrosome. PARPs are depicted in red and their binding partners/substrates are depicted in black. See section 1.1.3 for details.

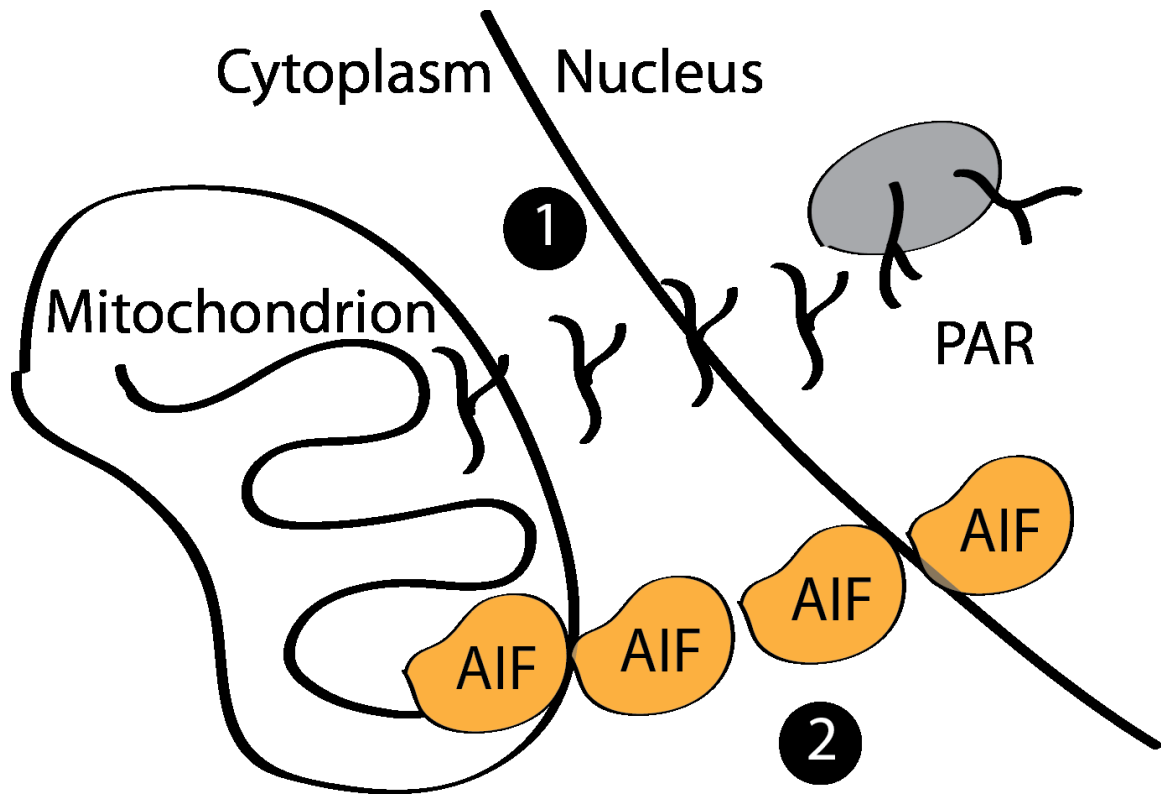


Figure 1-6 Parthanatos: PAR-induced cell death

Poly(ADP-ribose) has been implicated in cell death, termed Parthanatos: free PAR is released into the cytoplasm, triggering the release of apoptosis inducing factor (AIF), which then moves to the nucleus and initiates caspase-independent apoptosis [114].

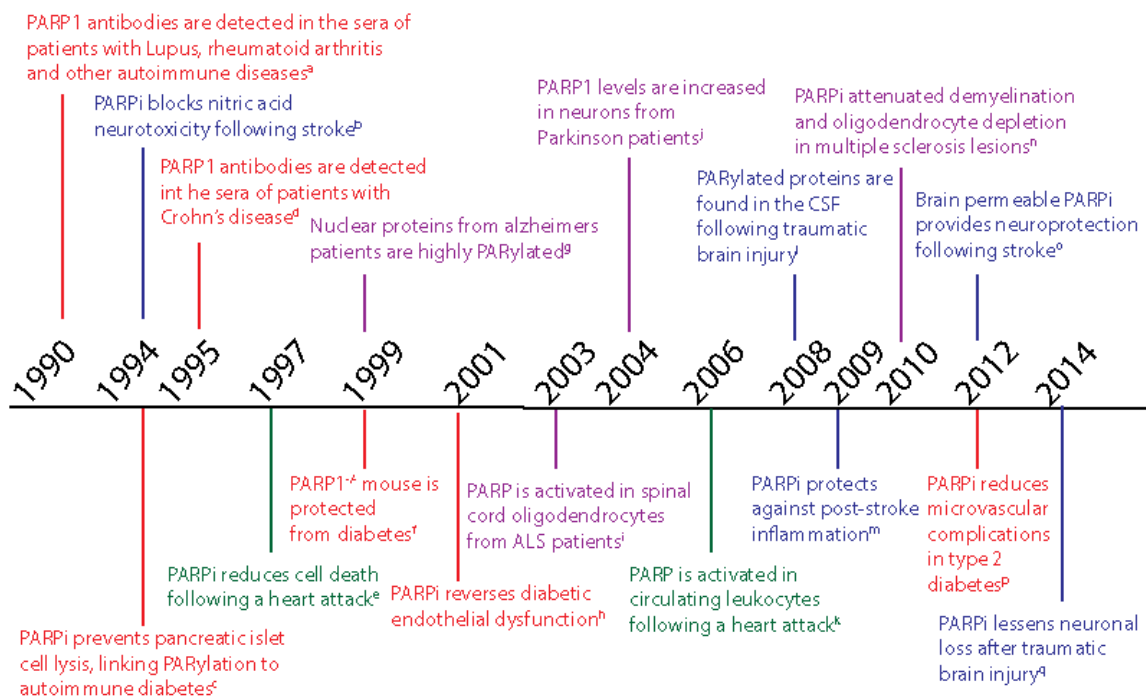


Figure 1-7 PARP inhibitors show therapeutic promise beyond cancer.

Findings from the last 25 years which have built a case for the exploration of PARP inhibitors for the treatment of neurotrauma (blue), neurodegeneration (purple), autoimmune disorders (red) and myocardial infarction (green). CSF = Cerebral Spinal Fluid, ALS = Amyotrophic Lateral Sclerosis, PARPi = PARP inhibitor. ^a[157]^b[158] ^c[105]^d[159] ^e[160]^f[161]^g[162]^h[163]ⁱ[164]^j[165] ^k[166]^l[167] ^m[168]ⁿ[169]^o[170]^p[171]^q[172].

1.5 References

1. Ciechanover, A., *Proteolysis: from the lysosome to ubiquitin and the proteasome*. Nat Rev Mol Cell Biol, 2005. **6**(1): p. 79-87.
2. Yeung, T., et al., *Membrane phosphatidylserine regulates surface charge and protein localization*. Science, 2008. **319**(5860): p. 210-3.
3. Pawson, T., G.D. Gish, and P. Nash, *SH2 domains, interaction modules and cellular wiring*. Trends Cell Biol, 2001. **11**(12): p. 504-11.
4. Smith, L.M. and N.L. Kelleher, *Proteoform: a single term describing protein complexity*. Nat Methods, 2013. **10**(3): p. 186-7.
5. Olsen, J.V. and M. Mann, *Status of large-scale analysis of post-translational modifications by mass spectrometry*. Mol Cell Proteomics, 2013. **12**(12): p. 3444-52.
6. Vyas, S., et al., *Family-wide analysis of poly(ADP-ribose) polymerase activity*. Nat Commun, 2014. **5**: p. 4426.
7. Chambon, P., J.D. Weill, and P. Mandel, *Nicotinamide mononucleotide activation of new DNA-dependent polyadenylic acid synthesizing nuclear enzyme*. Biochem Biophys Res Commun, 1963. **11**: p. 39-43.
8. Chambon, P., Weill, J.D., Doly, J., Strosser, M.T., Mandel, P., *On the formation of a novel adenylic compound by enzymatic extracts of liver nuclei*. Biochemical and Biophysical Research Communications, 1966. **25**(6): p. 638-643.
9. Benjamin, R.C. and D.M. Gill, *Poly(ADP-ribose) synthesis in vitro programmed by damaged DNA. A comparison of DNA molecules containing different types of strand breaks*. J Biol Chem, 1980. **255**(21): p. 10502-8.
10. Satoh, M.S., G.G. Poirier, and T. Lindahl, *Dual function for poly(ADP-ribose) synthesis in response to DNA strand breakage*. Biochemistry, 1994. **33**(23): p. 7099-106.
11. Wang, Z.Q., et al., *Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease*. Genes Dev, 1995. **9**(5): p. 509-20.
12. de Murcia, J.M., et al., *Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells*. Proc Natl Acad Sci U S A, 1997. **94**(14): p. 7303-7.
13. Masutani, M., et al., *Function of poly(ADP-ribose) polymerase in response to DNA damage: gene-disruption study in mice*. Mol Cell Biochem, 1999. **193**(1-2): p. 149-52.
14. Trucco, C., et al., *DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines*. Nucleic Acids Res, 1998. **26**(11): p. 2644-9.
15. Shieh, W.M., et al., *Poly(ADP-ribose) polymerase null mouse cells synthesize ADP-ribose polymers*. J Biol Chem, 1998. **273**(46): p. 30069-72.

16. Ame, J.C., et al., *PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase*. J Biol Chem, 1999. **274**(25): p. 17860-8.
17. Menissier de Murcia, J., et al., *Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse*. EMBO J, 2003. **22**(9): p. 2255-63.
18. Masson, M., et al., *XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage*. Mol Cell Biol, 1998. **18**(6): p. 3563-71.
19. El-Khamisy, S.F., et al., *A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage*. Nucleic Acids Res, 2003. **31**(19): p. 5526-33.
20. Okano, S., et al., *Spatial and temporal cellular responses to single-strand breaks in human cells*. Mol Cell Biol, 2003. **23**(11): p. 3974-81.
21. Schreiber, V., et al., *Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1*. J Biol Chem, 2002. **277**(25): p. 23028-36.
22. Wang, M., et al., *PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways*. Nucleic Acids Res, 2006. **34**(21): p. 6170-82.
23. Powell, S.N. and L.A. Kachnic, *Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation*. Oncogene, 2003. **22**(37): p. 5784-91.
24. Farmer, H., et al., *Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy*. Nature, 2005. **434**(7035): p. 917-21.
25. Bryant, H.E., et al., *Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase*. Nature, 2005. **434**(7035): p. 913-7.
26. Deeks, E.D., *Olaparib: first global approval*. Drugs, 2015. **75**(2): p. 231-40.
27. Boehler, C., et al., *Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression*. Proc Natl Acad Sci U S A, 2011. **108**(7): p. 2783-8.
28. Rulten, S.L., et al., *PARP-3 and APLF function together to accelerate nonhomologous end-joining*. Mol Cell, 2011. **41**(1): p. 33-45.
29. Yan, Q., et al., *BAL1 and its partner E3 ligase, BBAP, link Poly(ADP-ribose) activation, ubiquitylation, and double-strand DNA repair independent of ATM, MDC1, and RNF8*. Mol Cell Biol, 2013. **33**(4): p. 845-57.
30. Nicolae, C.M., et al., *The ADP-ribosyltransferase PARP10/ARTD10 interacts with proliferating cell nuclear antigen (PCNA) and is required for DNA damage tolerance*. J Biol Chem, 2014. **289**(19): p. 13627-37.

31. Nicolae, C.M., et al., *A novel role for the mono-ADP-ribosyltransferase PARP14/ARTD8 in promoting homologous recombination and protecting against replication stress*. Nucleic Acids Res, 2015. **43**(6): p. 3143-53.
32. Poirier, G.G., et al., *Poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure*. Proc Natl Acad Sci U S A, 1982. **79**(11): p. 3423-7.
33. Zahradka, P. and K. Ebisuzaki, *A shuttle mechanism for DNA-protein interactions. The regulation of poly(ADP-ribose) polymerase*. Eur J Biochem, 1982. **127**(3): p. 579-85.
34. Slattery, E., et al., *Purification and analysis of a factor which suppresses nick-induced transcription by RNA polymerase II and its identity with poly(ADP-ribose) polymerase*. J Biol Chem, 1983. **258**(9): p. 5955-9.
35. Cherney, B.W., et al., *cDNA sequence, protein structure, and chromosomal location of the human gene for poly(ADP-ribose) polymerase*. Proc Natl Acad Sci U S A, 1987. **84**(23): p. 8370-4.
36. Suzuki, H., et al., *Molecular cloning of cDNA for human poly(ADP-ribose) polymerase and expression of its gene during HL-60 cell differentiation*. Biochem Biophys Res Commun, 1987. **146**(2): p. 403-9.
37. Panzeter, P.L., C.A. Realini, and F.R. Althaus, *Noncovalent interactions of poly(adenosine diphosphate ribose) with histones*. Biochemistry, 1992. **31**(5): p. 1379-85.
38. Oei, S.L., et al., *Transcriptional regulation and autoregulation of the human gene for ADP-ribosyltransferase*. Mol Cell Biochem, 1994. **138**(1-2): p. 99-104.
39. Oei, S.L., et al., *Interaction of the transcription factor YY1 with human poly(ADP-ribosyl) transferase*. Biochem Biophys Res Commun, 1997. **240**(1): p. 108-11.
40. Oei, S.L., et al., *Regulation of RNA polymerase II-dependent transcription by poly(ADP-ribosyl)ation of transcription factors*. J Biol Chem, 1998. **273**(48): p. 31644-7.
41. Oei, S.L., et al., *A novel function of poly(ADP-ribosyl)ation: silencing of RNA polymerase II-dependent transcription*. Biochemistry, 1998. **37**(6): p. 1465-9.
42. Akiyama, T., et al., *Activation of Reg gene, a gene for insulin-producing beta -cell regeneration: poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly(ADP-ribosyl)ation*. Proc Natl Acad Sci U S A, 2001. **98**(1): p. 48-53.
43. Ju, B.G., et al., *Activating the PARP-1 sensor component of the groucho/TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway*. Cell, 2004. **119**(6): p. 815-29.

44. Tulin, A. and A. Spradling, *Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at Drosophila puff loci*. Science, 2003. **299**(5606): p. 560-2.
45. Kim, M.Y., et al., *NAD⁺-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1*. Cell, 2004. **119**(6): p. 803-14.
46. Petesch, S.J. and J.T. Lis, *Rapid, transcription-independent loss of nucleosomes over a large chromatin domain at Hsp70 loci*. Cell, 2008. **134**(1): p. 74-84.
47. Petesch, S.J. and J.T. Lis, *Activator-induced spread of poly(ADP-ribose) polymerase promotes nucleosome loss at Hsp70*. Mol Cell, 2012. **45**(1): p. 64-74.
48. Maeda, Y., et al., *PARP-2 interacts with TTF-1 and regulates expression of surfactant protein-B*. J Biol Chem, 2006. **281**(14): p. 9600-6.
49. Bai, P., et al., *Poly(ADP-ribose) polymerase-2 [corrected] controls adipocyte differentiation and adipose tissue function through the regulation of the activity of the retinoid X receptor/ peroxisome proliferator-activated receptor-gamma [corrected] heterodimer*. J Biol Chem, 2007. **282**(52): p. 37738-46.
50. Bai, P., et al., *PARP-2 regulates SIRT1 expression and whole-body energy expenditure*. Cell Metab, 2011. **13**(4): p. 450-60.
51. Malanga, M. and F.R. Althaus, *Poly(ADP-ribose) reactivates stalled DNA topoisomerase I and Induces DNA strand break resealing*. J Biol Chem, 2004. **279**(7): p. 5244-8.
52. Meyer-Ficca, M.L., et al., *Poly(ADP-ribose) polymerases PARP1 and PARP2 modulate topoisomerase II beta (TOP2B) function during chromatin condensation in mouse spermiogenesis*. Biol Reprod, 2011. **84**(5): p. 900-9.
53. Wittmann, T., A. Hyman, and A. Desai, *The spindle: a dynamic assembly of microtubules and motors*. Nat Cell Biol, 2001. **3**(1): p. E28-34.
54. Smith, S., et al., *Tankyrase, a poly(ADP-ribose) polymerase at human telomeres*. Science, 1998. **282**(5393): p. 1484-7.
55. Cook, B.D., et al., *Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres*. Mol Cell Biol, 2002. **22**(1): p. 332-42.
56. Dynek, J.N. and S. Smith, *Resolution of sister telomere association is required for progression through mitosis*. Science, 2004. **304**(5667): p. 97-100.
57. Donigian, J.R. and T. de Lange, *The role of the poly(ADP-ribose) polymerase tankyrase1 in telomere length control by the TRF1 component of the shelterin complex*. J Biol Chem, 2007. **282**(31): p. 22662-7.

58. Dantzer, F., et al., *Functional interaction between poly(ADP-Ribose) polymerase 2 (PARP-2) and TRF2: PARP activity negatively regulates TRF2*. Mol Cell Biol, 2004. **24**(4): p. 1595-607.
59. Gomez, M., et al., *PARP1 Is a TRF2-associated poly(ADP-ribose)polymerase and protects eroded telomeres*. Mol Biol Cell, 2006. **17**(4): p. 1686-96.
60. Earle, E., et al., *Poly(ADP-ribose) polymerase at active centromeres and neocentromeres at metaphase*. Hum Mol Genet, 2000. **9**(2): p. 187-94.
61. Saxena, A., et al., *Poly(ADP-ribose) polymerase 2 localizes to mammalian active centromeres and interacts with PARP-1, Cenpa, Cenpb and Bub3, but not Cenpc*. Hum Mol Genet, 2002. **11**(19): p. 2319-29.
62. Saxena, A., et al., *Centromere proteins Cenpa, Cenpb, and Bub3 interact with poly(ADP-ribose) polymerase-1 protein and are poly(ADP-ribosyl)ated*. J Biol Chem, 2002. **277**(30): p. 26921-6.
63. Shall, S. and G. de Murcia, *Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model?* Mutat Res, 2000. **460**(1): p. 1-15.
64. Kameoka, M., et al., *Poly(ADP-ribose)polymerase-1 is required for integration of the human immunodeficiency virus type 1 genome near centromeric alphoid DNA in human and murine cells*. Biochem Biophys Res Commun, 2005. **334**(2): p. 412-7.
65. Yang, F., C. Baumann, and R. De La Fuente, *Persistence of histone H2AX phosphorylation after meiotic chromosome synapsis and abnormal centromere cohesion in poly (ADP-ribose) polymerase (Parp-1) null oocytes*. Dev Biol, 2009. **331**(2): p. 326-38.
66. Smith, S. and T. de Lange, *Cell cycle dependent localization of the telomeric PARP, tankyrase, to nuclear pore complexes and centrosomes*. J Cell Sci, 1999. **112 (Pt 21)**: p. 3649-56.
67. Kickhoefer, V.A., et al., *The 193-kD vault protein, VPARP, is a novel poly(ADP-ribose) polymerase*. J Cell Biol, 1999. **146**(5): p. 917-28.
68. Augustin, A., et al., *PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression*. J Cell Sci, 2003. **116**(Pt 8): p. 1551-62.
69. Chang, P., M.K. Jacobson, and T.J. Mitchison, *Poly(ADP-ribose) is required for spindle assembly and structure*. Nature, 2004. **432**(7017): p. 645-9.
70. Chang, P., M. Coughlin, and T.J. Mitchison, *Tankyrase-1 polymerization of poly(ADP-ribose) is required for spindle structure and function*. Nat Cell Biol, 2005. **7**(11): p. 1133-9.
71. Chang, W., J.N. Dynek, and S. Smith, *NuMA is a major acceptor of poly(ADP-ribosyl)ation by tankyrase 1 in mitosis*. Biochem J, 2005. **391**(Pt 2): p. 177-84.

72. Vyas, S., et al., *A systematic analysis of the PARP protein family identifies new functions critical for cell physiology*. Nat Commun, 2013. **4**: p. 2240.
73. Roberts, J.H., et al., *Cytoplasmic poly(ADP-ribose) polymerase during the HeLa cell cycle*. Arch Biochem Biophys, 1975. **171**(1): p. 305-15.
74. Elkaim, R., et al., *Adenosine diphosphate ribosyltransferase and protein acceptors associated with cytoplasmic free messenger ribonucleoprotein particles*. Biochimie, 1983. **65**(11-12): p. 653-9.
75. Thomassin, H., C. Niedergang, and P. Mandel, *Characterization of the poly(ADP-ribose) polymerase associated with free cytoplasmic mRNA-protein particles*. Biochem Biophys Res Commun, 1985. **133**(2): p. 654-61.
76. Leung, A.K., et al., *Poly(ADP-ribose) regulates stress responses and microRNA activity in the cytoplasm*. Mol Cell, 2011. **42**(4): p. 489-99.
77. Kostka, G. and A. Schweiger, *ADP-ribosylation of proteins associated with heterogeneous nuclear RNA in rat liver nuclei*. Biochim Biophys Acta, 1982. **696**(2): p. 139-44.
78. Prasad, S., J. Walent, and A. Dritschilo, *ADP-ribosylation of heterogeneous ribonucleoproteins in HeLa cells*. Biochem Biophys Res Commun, 1994. **204**(2): p. 772-9.
79. Sitikov, A.S., et al., *Eukaryotic elongation factor 2 loses its non-specific affinity for RNA and leaves polyribosomes as a result of ADP-ribosylation*. FEBS Lett, 1984. **176**(2): p. 406-10.
80. Gagne, J.P., et al., *Quantitative proteomics profiling of the poly(ADP-ribose)-related response to genotoxic stress*. Nucleic Acids Res, 2012. **40**(16): p. 7788-805.
81. Gagne, J.P., et al., *Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes*. Nucleic Acids Res, 2008. **36**(22): p. 6959-76.
82. Isabelle, M., et al., *Quantitative proteomics and dynamic imaging reveal that G3BP-mediated stress granule assembly is poly(ADP-ribose)-dependent following exposure to MNNG-induced DNA alkylation*. J Cell Sci, 2012. **125**(Pt 19): p. 4555-66.
83. Jungmichel, S., et al., *Proteome-wide Identification of Poly(ADP-Ribosyl)ation Targets in Different Genotoxic Stress Responses*. Mol Cell, 2013.
84. Daniels, C.M., S.E. Ong, and A.K. Leung, *A phosphoproteomic approach to characterize protein mono and poly(ADP-ribosyl)ation sites from whole cell lysate*. J Proteome Res, 2014.
85. Zhang, Y., et al., *Site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome*. Nat Methods, 2013. **10**(10): p. 981-4.
86. Daniels, C.M., S.E. Ong, and A.K. Leung, *The Promise of Proteomics for the Study of ADP-Ribosylation*. Mol Cell, 2015. **58**(6): p. 911-24.

87. Leung, A.K., *Poly(ADP-ribose): an organizer of cellular architecture*. J Cell Biol, 2014. **205**(5): p. 613-9.
88. Gao, G., X. Guo, and S.P. Goff, *Inhibition of retroviral RNA production by ZAP, a CCH-type zinc finger protein*. Science, 2002. **297**(5587): p. 1703-6.
89. Bick, M.J., et al., *Expression of the zinc-finger antiviral protein inhibits alphavirus replication*. J Virol, 2003. **77**(21): p. 11555-62.
90. Guo, X., et al., *The zinc finger antiviral protein directly binds to specific viral mRNAs through the CCH zinc finger motifs*. J Virol, 2004. **78**(23): p. 12781-7.
91. Zhu, Y., et al., *Zinc-finger antiviral protein inhibits HIV-1 infection by selectively targeting multiply spliced viral mRNAs for degradation*. Proc Natl Acad Sci U S A, 2011. **108**(38): p. 15834-9.
92. Atasheva, S., et al., *New PARP gene with an anti-alphavirus function*. J Virol, 2012. **86**(15): p. 8147-60.
93. Atasheva, S., E.I. Frolova, and I. Frolov, *Interferon-stimulated poly(ADP-Ribose) polymerases are potent inhibitors of cellular translation and virus replication*. J Virol, 2014. **88**(4): p. 2116-30.
94. Soldani, C. and A.I. Scovassi, *Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update*. Apoptosis, 2002. **7**(4): p. 321-8.
95. Kaufmann, S.H., et al., *Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis*. Cancer Res, 1993. **53**(17): p. 3976-85.
96. Berger, N.A., *Poly(ADP-ribose) in the cellular response to DNA damage*. Radiat Res, 1985. **101**(1): p. 4-15.
97. Herceg, Z. and Z.Q. Wang, *Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis*. Mol Cell Biol, 1999. **19**(7): p. 5124-33.
98. Oliver, F.J., et al., *Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant*. J Biol Chem, 1998. **273**(50): p. 33533-9.
99. Halappanavar, S.S., et al., *Survival and proliferation of cells expressing caspase-uncleavable Poly(ADP-ribose) polymerase in response to death-inducing DNA damage by an alkylating agent*. J Biol Chem, 1999. **274**(52): p. 37097-104.
100. Bae, J., J.R. Donigian, and A.J. Hsueh, *Tankyrase 1 interacts with Mcl-1 proteins and inhibits their regulation of apoptosis*. J Biol Chem, 2003. **278**(7): p. 5195-204.
101. Lu, H., et al., *Silencing tankyrase and telomerase promotes A549 human lung adenocarcinoma cell apoptosis and inhibits proliferation*. Oncol Rep, 2013. **30**(4): p. 1745-52.

102. Tian, X.H., et al., *XAV939, a tankyrase 1 inhibitor, promotes cell apoptosis in neuroblastoma cell lines by inhibiting Wnt/beta-catenin signaling pathway*. J Exp Clin Cancer Res, 2013. **32**: p. 100.
103. Stratford, E.W., et al., *The tankyrase-specific inhibitor JW74 affects cell cycle progression and induces apoptosis and differentiation in osteosarcoma cell lines*. Cancer Med, 2014. **3**(1): p. 36-46.
104. Sims, J.L., S.J. Berger, and N.A. Berger, *Poly(ADP-ribose) Polymerase inhibitors preserve nicotinamide adenine dinucleotide and adenosine 5'-triphosphate pools in DNA-damaged cells: mechanism of stimulation of unscheduled DNA synthesis*. Biochemistry, 1983. **22**(22): p. 5188-94.
105. Radons, J., et al., *Nitric oxide toxicity in islet cells involves poly(ADP-ribose) polymerase activation and concomitant NAD⁺ depletion*. Biochem Biophys Res Commun, 1994. **199**(3): p. 1270-7.
106. Eliasson, M.J., et al., *Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia*. Nat Med, 1997. **3**(10): p. 1089-95.
107. Ha, H.C. and S.H. Snyder, *Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion*. Proc Natl Acad Sci U S A, 1999. **96**(24): p. 13978-82.
108. Los, M., et al., *Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling*. Mol Biol Cell, 2002. **13**(3): p. 978-88.
109. Ditsworth, D., W.X. Zong, and C.B. Thompson, *Activation of poly(ADP-ribose) polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus*. J Biol Chem, 2007. **282**(24): p. 17845-54.
110. Yang, Z., et al., *PARP-1 mediates LPS-induced HMGB1 release by macrophages through regulation of HMGB1 acetylation*. J Immunol, 2014. **193**(12): p. 6114-23.
111. Kaminker, P.G., et al., *TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression*. J Biol Chem, 2001. **276**(38): p. 35891-9.
112. Galluzzi, L., et al., *Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012*. Cell Death Differ, 2012. **19**(1): p. 107-20.
113. Yu, S.W., et al., *Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor*. Science, 2002. **297**(5579): p. 259-63.
114. Yu, S.W., et al., *Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death*. Proc Natl Acad Sci U S A, 2006. **103**(48): p. 18314-9.

115. Wang, Y., et al., *Poly(ADP-ribose) (PAR) binding to apoptosis-inducing factor is critical for PAR polymerase-1-dependent cell death (parthanatos)*. Sci Signal, 2011. **4**(167): p. ra20.
116. Andrabi, S.A., et al., *Iduna protects the brain from glutamate excitotoxicity and stroke by interfering with poly(ADP-ribose) polymer-induced cell death*. Nat Med, 2011. **17**(6): p. 692-9.
117. Weltin, D., et al., *Immunosuppressive activities of 6(5H)-phenanthridinone, a new poly(ADP-ribose)polymerase inhibitor*. Int J Immunopharmacol, 1995. **17**(4): p. 265-71.
118. Oliver, F.J., et al., *Resistance to endotoxic shock as a consequence of defective NF-kappaB activation in poly (ADP-ribose) polymerase-1 deficient mice*. EMBO J, 1999. **18**(16): p. 4446-54.
119. Hassa, P.O. and M.O. Hottiger, *A role of poly (ADP-ribose) polymerase in NF-kappaB transcriptional activation*. Biol Chem, 1999. **380**(7-8): p. 953-9.
120. Hinz, M., et al., *A cytoplasmic ATM-TRAF6-clAP1 module links nuclear DNA damage signaling to ubiquitin-mediated NF-kappaB activation*. Mol Cell, 2010. **40**(1): p. 63-74.
121. Stilmann, M., et al., *A nuclear poly(ADP-ribose)-dependent signalosome confers DNA damage-induced IkappaB kinase activation*. Mol Cell, 2009. **36**(3): p. 365-78.
122. Zerfaoui, M., et al., *Poly(ADP-ribose) polymerase-1 is a determining factor in Crm1-mediated nuclear export and retention of p65 NF-kappa B upon TLR4 stimulation*. J Immunol, 2010. **185**(3): p. 1894-902.
123. Hunter, J.E., et al., *NF-kappaB mediates radio-sensitization by the PARP-1 inhibitor, AG-014699*. Oncogene, 2012. **31**(2): p. 251-64.
124. Goenka, S., S.H. Cho, and M. Boothby, *Collaborator of Stat6 (CoaSt6)-associated poly(ADP-ribose) polymerase activity modulates Stat6-dependent gene transcription*. J Biol Chem, 2007. **282**(26): p. 18732-9.
125. Mehrotra, P., et al., *PARP-14 functions as a transcriptional switch for Stat6-dependent gene activation*. J Biol Chem, 2011. **286**(3): p. 1767-76.
126. Beck, C., et al., *Poly(ADP-ribose) polymerases in double-strand break repair: focus on PARP1, PARP2 and PARP3*. Exp Cell Res, 2014. **329**(1): p. 18-25.
127. Liu, J.F., P.A. Konstantinopoulos, and U.A. Matulonis, *PARP inhibitors in ovarian cancer: current status and future promise*. Gynecol Oncol, 2014. **133**(2): p. 362-9.
128. Lord, C.J., A.N. Tutt, and A. Ashworth, *Synthetic Lethality and Cancer Therapy: Lessons Learned from the Development of PARP Inhibitors*. Annu Rev Med, 2014.

129. O'Sullivan, C.C., et al., *Beyond Breast and Ovarian Cancers: PARP Inhibitors for BRCA Mutation-Associated and BRCA-Like Solid Tumors*. Front Oncol, 2014. **4**: p. 42.
130. Curtin, N.J. and C. Szabo, *Therapeutic applications of PARP inhibitors: anticancer therapy and beyond*. Mol Aspects Med, 2013. **34**(6): p. 1217-56.
131. Bensimon, A., A.J. Heck, and R. Aebersold, *Mass spectrometry-based proteomics and network biology*. Annu Rev Biochem, 2012. **81**: p. 379-405.
132. Aebersold, R. and M. Mann, *Mass spectrometry-based proteomics*. Nature, 2003. **422**(6928): p. 198-207.
133. Olsen, J.V., et al., *Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis*. Sci Signal, 2010. **3**(104): p. ra3.
134. Yu, Y., et al., *Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling*. Science, 2011. **332**(6035): p. 1322-6.
135. Xu, G., J.S. Paige, and S.R. Jaffrey, *Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling*. Nat Biotechnol, 2010. **28**(8): p. 868-73.
136. Choudhary, C., et al., *Lysine acetylation targets protein complexes and co-regulates major cellular functions*. Science, 2009. **325**(5942): p. 834-40.
137. Zhang, H., et al., *Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry*. Nat Biotechnol, 2003. **21**(6): p. 660-6.
138. Hottiger, M.O., et al., *Toward a unified nomenclature for mammalian ADP-ribosyltransferases*. Trends Biochem Sci, 2010. **35**(4): p. 208-19.
139. Otto, H., et al., *In silico characterization of the family of PARP-like poly(ADP-ribosyl)transferases (pARTs)*. BMC Genomics, 2005. **6**: p. 139.
140. Roitt, I.M., *The inhibition of carbohydrate metabolism in ascites-tumour cells by ethyleneimines*. Biochem J, 1956. **63**(2): p. 300-7.
141. Durkacz, B.W., et al., *(ADP-ribose)_n participates in DNA excision repair*. Nature, 1980. **283**(5747): p. 593-6.
142. Ben-Hur, E., C.C. Chen, and M.M. Elkind, *Inhibitors of poly(adenosine diphosphoribose) synthetase, examination of metabolic perturbations, and enhancement of radiation response in Chinese hamster cells*. Cancer Res, 1985. **45**(5): p. 2123-7.
143. Banasik, M., et al., *Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase*. J Biol Chem, 1992. **267**(3): p. 1569-75.
144. Bernges, F. and W.J. Zeller, *Combination effects of poly(ADP-ribose) polymerase inhibitors and DNA-damaging agents in ovarian tumor cell*

- lines--with special reference to cisplatin.* J Cancer Res Clin Oncol, 1996. **122**(11): p. 665-70.
145. McCabe, N., et al., *Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition.* Cancer Res, 2006. **66**(16): p. 8109-15.
 146. Evers, B., et al., *Selective inhibition of BRCA2-deficient mammary tumor cell growth by AZD2281 and cisplatin.* Clin Cancer Res, 2008. **14**(12): p. 3916-25.
 147. Rottenberg, S., et al., *High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs.* Proc Natl Acad Sci U S A, 2008. **105**(44): p. 17079-84.
 148. Mendes-Pereira, A.M., et al., *Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors.* EMBO Mol Med, 2009. **1**(6-7): p. 315-22.
 149. Fong, P.C., et al., *Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers.* N Engl J Med, 2009. **361**(2): p. 123-34.
 150. Tutt, A., et al., *Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial.* Lancet, 2010. **376**(9737): p. 235-44.
 151. Audeh, M.W., et al., *Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: a proof-of-concept trial.* Lancet, 2010. **376**(9737): p. 245-51.
 152. O'Shaughnessy, J., et al., *Iniparib plus chemotherapy in metastatic triple-negative breast cancer.* N Engl J Med, 2011. **364**(3): p. 205-14.
 153. Goncalves, A., et al., *Poly(ADP-ribose) polymerase-1 mRNA expression in human breast cancer: a meta-analysis.* Breast Cancer Res Treat, 2011. **127**(1): p. 273-81.
 154. Wahlberg, E., et al., *Family-wide chemical profiling and structural analysis of PARP and tankyrase inhibitors.* Nat Biotechnol, 2012. **30**(3): p. 283-8.
 155. Liu, X., et al., *Iniparib nonselectively modifies cysteine-containing proteins in tumor cells and is not a bona fide PARP inhibitor.* Clin Cancer Res, 2012. **18**(2): p. 510-23.
 156. Murai, J., et al., *Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors.* Cancer Res, 2012. **72**(21): p. 5588-99.
 157. Negri, C., et al., *Autoantibodies to poly(ADP-ribose)polymerase in autoimmune diseases.* Autoimmunity, 1990. **6**(3): p. 203-9.
 158. Zhang, J., et al., *Nitric oxide activation of poly(ADP-ribose) synthetase in neurotoxicity.* Science, 1994. **263**(5147): p. 687-9.
 159. Reumaux, D., et al., *Distinct production of autoantibodies to nuclear components in ulcerative colitis and in Crohn's disease.* Clin Immunol Immunopathol, 1995. **77**(3): p. 349-57.

160. Thiernemann, C., et al., *Inhibition of the activity of poly(ADP ribose) synthetase reduces ischemia-reperfusion injury in the heart and skeletal muscle*. Proc Natl Acad Sci U S A, 1997. **94**(2): p. 679-83.
161. Pieper, A.A., et al., *Poly(ADP-ribose) polymerase-deficient mice are protected from streptozotocin-induced diabetes*. Proc Natl Acad Sci U S A, 1999. **96**(6): p. 3059-64.
162. Love, S., R. Barber, and G.K. Wilcock, *Increased poly(ADP-ribosylation) of nuclear proteins in Alzheimer's disease*. Brain, 1999. **122 (Pt 2)**: p. 247-53.
163. Soriano, F.G., et al., *Rapid reversal of the diabetic endothelial dysfunction by pharmacological inhibition of poly(ADP-ribose) polymerase*. Circ Res, 2001. **89**(8): p. 684-91.
164. Kim, S.H., et al., *PARP expression is increased in astrocytes but decreased in motor neurons in the spinal cord of sporadic ALS patients*. J Neuropathol Exp Neurol, 2003. **62**(1): p. 88-103.
165. Soos, J., et al., *The expression of PARP, NF-kappa B and parvalbumin is increased in Parkinson disease*. Neuroreport, 2004. **15**(11): p. 1715-8.
166. Toth-Zsamboki, E., et al., *Activation of poly(ADP-ribose) polymerase by myocardial ischemia and coronary reperfusion in human circulating leukocytes*. Mol Med, 2006. **12**(9-10): p. 221-8.
167. Fink, E.L., et al., *Quantification of poly(ADP-ribose)-modified proteins in cerebrospinal fluid from infants and children after traumatic brain injury*. J Cereb Blood Flow Metab, 2008. **28**(9): p. 1523-9.
168. Kauppinen, T.M., et al., *Inhibition of poly(ADP-ribose) polymerase suppresses inflammation and promotes recovery after ischemic injury*. J Cereb Blood Flow Metab, 2009. **29**(4): p. 820-9.
169. Veto, S., et al., *Inhibiting poly(ADP-ribose) polymerase: a potential therapy against oligodendrocyte death*. Brain, 2010. **133**(Pt 3): p. 822-34.
170. Moroni, F., et al., *Long-lasting neuroprotection and neurological improvement in stroke models with new, potent and brain permeable inhibitors of poly(ADP-ribose) polymerase*. Br J Pharmacol, 2012. **165**(5): p. 1487-500.
171. Choi, S.K., et al., *Poly(ADP-ribose) polymerase 1 inhibition improves coronary arteriole function in type 2 diabetes mellitus*. Hypertension, 2012. **59**(5): p. 1060-8.
172. Stoica, B.A., et al., *PARP-1 inhibition attenuates neuronal loss, microglia activation and neurological deficits after traumatic brain injury*. J Neurotrauma, 2014. **31**(8): p. 758-72.

2 Phosphoproteomic Approach to Characterize Protein Mono- and Poly(ADP-ribosyl)ation Sites from Cells

Casey M. Daniels¹, Shao-En Ong², and Anthony K.L. Leung¹

¹Department of Biochemistry and Molecular Biology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205, United States

²Department of Pharmacology, University of Washington, Seattle, Washington 98195, United States

Published as:

Phosphoproteomic Approach to Characterize Protein Mono- and Poly(ADP-ribosyl)ation Sites from Cells

Casey M. Daniels, Shao-En Ong, and Anthony K. L. Leung

Journal of Proteome Research 2014 13 (8), 3510-3522

DOI: 10.1021/pr401032q

2.1 Abstract

Poly(ADP-ribose), or PAR, is a cellular polymer implicated in DNA/RNA metabolism, cell death, and cellular stress response via its role as a post-translational modification, signaling molecule, and scaffolding element. PAR is synthesized by a family of proteins known as Poly(ADP-ribose) Polymerases, or PARPs, which attach PAR polymers to various amino acids of substrate proteins. The nature of these polymers (large, charged, heterogeneous, base-labile) has made these attachment sites difficult to study by mass spectrometry. Here we propose a new pipeline which allows for the identification of mono(ADP-ribosyl)ation and poly(ADP-ribosyl)ation sites via the enzymatic product of phosphodiesterase-treated ADP-ribose, or phospho(ribose). The power of this method lies in the enrichment potential of phospho(ribose) – which we show to be enriched by phosphoproteomic techniques when a neutral buffer, which allows for retention of the base-labile attachment site, is used for elution. Through the identification of PARP-1 in vitro automodification sites as well as endogenous ADP-ribosylation sites from whole cells we have shown ADP-ribose can exist on adjacent amino acid residues as well as both lysine and arginine in addition to known acidic modification sites. The universality of this technique has allowed us to show that enrichment of ADP-ribosylated proteins by macrodomain leads to a bias against ADP-ribose modifications conjugated to glutamic acids, suggesting that the macrodomain is either removing or selecting against these distinct protein attachments. Ultimately, the enrichment pipeline presented here offers a universal approach for characterizing the mono and poly(ADP-ribosyl)ated proteome.

2.2 Introduction

ADP-ribose (ADPr) is a post-translational modification which is synthesized by a family of ADP-ribosyltransferases[1], commonly known as Poly(ADP-ribose) Polymerases, or PARPs. These modifications are derived from the hydrolysis of NAD⁺ and exist in both the monomeric and polymeric form, the latter of which is made up of 2-200 ADP-ribose subunits. The canonical role for this polymer has been in the identification and repair of DNA nicks and double-stranded breaks via activation of the founding member of the PARP family, PARP-1[2]. Indeed, this role has ushered in

PARP-1 as a chemotherapeutic target, as the loss of PARP-1 sensitizes cells to genomic assault by established chemotherapeutic and radiation-based treatment[3]. It is worth noting, however, that PAR's cellular role has expanded beyond DNA repair into regulation of (among others) apoptosis[4], chromatin structure[5], synthesis of DNA/RNA[6, 7], telomere maintenance[8], protein degradation[9], and microRNA activities[10]. Not surprisingly, the increase in understanding of PAR's biological roles has led to recognition of its therapeutic potential beyond modulation of DNA damage, including the treatment of necrosis and inflammation[11]. PAR's relative, mono(ADP-ribose), is far less studied but has received increasing attention due to a number of recent studies which have identified the enzymes which reverse mono(ADP-ribosylation) as well as novel roles for mono(ADP-ribose) in the cell[12]. In an effort to aid in the understanding of the mono and poly(ADP-ribosyl)ated proteome we have looked to mass spectrometry to define the molecular basis of ADP-ribosylation, and will begin by characterizing the poly(ADP-ribosylation) (or PARylation) activity of human PARP-1 (hPARP-1).

The hurdles which have kept mass spectrometry and proteomics from becoming universal tools for studying PARylation have to do with the physical properties of the modification itself: firstly, the modification can expand linearly or by branching and vary dramatically in length, resulting in a large, heterogeneous polymer without a defined mass. Secondly, many of the amino acid attachment sites are base-labile[13], preventing researchers from exposing the modified proteins or peptides to basic solutions which are commonly used in proteomic sample preparations. Finally, the modification is dynamic, with basal levels existing below the level of detection of most molecular tools used in proteomics. One recently published approach to identify ADP-ribosylation sites by mass spectrometry paired boronate enrichment of ADP-ribosylated proteins with subsequent release of mono and poly(ADP-ribose) from substrates by hydroxylamine[14]. This elution strategy breaks ester bonds between the ADP-ribose subunits and the carboxyl groups of aspartate and glutamate residues, leaving a characteristic 15.01 Da mass signature on the modified residue. Notably, this approach cannot identify non-acidic ADP-ribosylated residues and up to 33% of total ADP-

ribosylated amino acid residues have been shown to be hydroxylamine-insensitive[15]. In particular lysine residues are important for the *in vitro* and *in vivo* activation of PARP-1[16, 17], as well as substrate regulation by PARPs, for example chromatin remodeling via PARylation of the lysine residues on histone tails[18].

Because a global approach to identify all possible ADP-ribosylation sites is still needed we have developed an enrichment protocol based on the digestion of ADP-ribose by snake venom phosphodiesterase (SVP), a pyrophosphatase which cleaves ADPr subunits down to phospho(ribose) and 5'-AMP[19]. This digestion produces a single phospho(ribose) group at the site of modification which can be identified by mass spectrometry as an adduct of 212.01 daltons[20]. Given the similarity of phospho(ribosyl) and phosphate groups, we reasoned that existing phosphoproteomic techniques may be used to enrich phospho(ribosyl)ated peptides. Indeed a 2010 phosphoenrichment study which utilized immobilized metal affinity chromatography (IMAC) to enrich phosphopeptides was searched in 2012 for a co-enrichment of ADP-ribose and/or phosphoribose, both of which were found to have been enriched[21, 22]. More recently, Chapman et al. demonstrated the feasibility of this approach to identify PARylation sites on a purified, automodified human PARP-1[23]. Here we have independently tested and validated this approach to identify ADP-ribosylation sites; we further compared three commercially available phosphoenrichment matrices and their use in enriching and characterizing phospho(ribosyl)ated peptides of hPARP-1 from a complex background of HeLa whole cell lysate. Finally we have demonstrated the application of this method to identify endogenous mono and poly(ADP-ribosyl)ation sites by mass spectrometry, yielding both known and novel acceptors of ADP-ribose, including a number which identify ADP-ribose on arginine residues.

2.3 Materials and Methods

Expression and purification of HisPARP-1

Method was adapted from Langelier *et al.* [24], briefly: 6 liters of His-PARP-1 expressing DE3 cells were lysed in a cell homogenizer in the presence of 0.1% NP-40, 20 U/mL DNase I, 5 mM MgCl₂, 1

μ M bestatin, 1 μ M pepstatin A, and 1x Roche cOmplete EDTA-free protease inhibitor. Lysate was cleared by centrifugation and loaded onto an ÄKTA FPLC (GE, 18-1900-26) with a pre-equilibrated 5 mL HisTrap FF Crude column (GE, 17-5286-01) where it was washed with 10 column volumes of loading buffer (20 mM NaPO₄ pH 7.4, 1M NaCl, 0.5 mM TCEP, 40 mM imidazole pH 7.4, 1% glycerol, 1x Roche cOmplete EDTA-free protease inhibitor) before being eluted in 2 column volumes of elution buffer (20 mM NaPO₄ pH 7.4, 0.5 M NaCl, 0.5 mM TCEP, 0.5 M imidazole pH 7.4, 1% glycerol). Eluted samples were diluted 1:1 in heparin no-salt buffer (50 mM Tris pH 7.0, 0.1 mM Tris-(2-Carboxyethyl)phosphine, 1% glycerol) and loaded onto a pre-equilibrated 5 mL heparin column (GE, 17-0407-01), washed with 5 volumes of low-salt buffer (50 mM Tris pH 7, 0.1 mM TCEP, 250 mM NaCl) and eluted over a gradient from 0-70% high-salt buffer (50 mM Tris pH 7, 0.1 mM TCEP, 1M NaCl, 1% glycerol). Desired fractions were pooled and concentrated using a spin concentrator (30,000 MWCO, Amicon Z648035) before being loaded onto a pre-equilibrated size exclusion column (GE, Superdex 200/10/300 GL) in size purification buffer (25 mM HEPES pH 8, 0.1 mM TCEP, 150 mM NaCl), desired fractions were pooled and stored at -80°C. All FPLC results were analyzed with UNICORN 5.01 (Build 318).

Purification of Snake Venom Phosphodiesterase I (SVP)

Protocol was adapted from Oka *et al*[25], briefly: (2) 100 unit vials of *Crotalus adamanteus* Phosphodiesterase I (Worthington, LS003926) were dissolved into 1 mL of loading buffer (10 mM Tris-Cl pH 7.5, 50 mM NaCl, 10% glycerol) and then loaded onto a pre-equilibrated 1 mL HiTrap blue sepharose column (GE, 17-0412-01), washed with 5 column volumes of loading buffer and then 5 column volumes of elution buffer (10 mM Tris-Cl pH 7.5, 50 mM NaCl, 10% glycerol, 150 mM potassium phosphate). Desired fractions were pooled, dialyzed against loading buffer, and stored at -80°C. If enzyme preps were to be used to treat denatured proteins an additional purification was needed to remove any contaminating proteases: samples were dialyzed into size exclusion chromatography buffer (10 mM Tris-Cl pH 7.3, 50 mM NaCl, 15 mM MgCl₂, 1% glycerol) and

resolved over a SuperDex 200/10/300 GL (GE Healthcare) using an ÄKTA FPLC (GE, 18-1900-26), desired fractions were pooled and stored at -80°C. All FPLC results were analyzed with UNICORN 5.01 (Build 318).

Preparing oligos for *in vitro* PARP-1 activation

Oligo sequences were from Langelier *et al* [24]. Forward (GGGTGGCGCCGCTTGGG) and reverse (CCCAAGCGGCCGCAACCC) oligos were mixed 1:1 in H₂O, heated to 95°C for 2 minutes and then ramp cooled to 25°C over 45 minutes.

Automodification of HisPARP-1 *in vitro*

HisPARP-1 was attached to Promega MagneHis beads (1 µg PARP-1/µL beads/5 µL attachment buffer) for 2 hours at 4°C in attachment buffer (50 mM Tris pH 7.4, 1% Tween, 0.2mM DTT, 10% glycerol, 10 mM MgCl₂). Beads were washed twice with 100 µL wash buffer (50 mM sodium phosphate buffer pH 7.4, 200 mM NaCl, 5 mM imidazole pH 7.4) and then exposed to 30 µM (0.6% hot) ³²P β-NAD⁺ in automodification buffer (20 mM Tris pH 7.5, 50 mM NaCl, 50 µM TCEP, 5 mM MgCl₂, 1.2 µM annealed DNA) for 10 minutes followed by a chase of 2 mM cold β-NAD⁺ for 60 minutes, all at 25°C rotating at 500rpm. For SDS-PAGE beads were washed twice in 100 µL wash buffer and eluted into 15 µL of 1x SDS-PAGE buffer, separated on an in-house 6-10% SDS-PAGE gel, fixed overnight (50% methanol, 10% acetic acid), washed for 30 minutes (H₂O), stained with Pro-Q Diamond Phosphoprotein stain (Invitrogen, MP 33300) for 1 hour, destained for 3x30 minutes (20% acetonitrile, 50 mM sodium acetate pH 4), washed for 10 minutes (H₂O) and imaged on a Fuji FLA7000 (filter: O580, wavelength: 532nm). Pro-Q Diamond staining was validated based on comparison to Pro-Q Diamond PeppermintStick ladder (Life Technologies, P27167). Total protein was determined by Coomassie Blue staining (Invitrogen LC6060) and ³²P-labeling was determined by overnight exposure against a phosphor-screen (GE, BAS-III 2040) followed by imaging on a Fuji FLA7000 (IP). Western blotting for poly(ADP-ribose) was performed by transferring proteins (Invitrogen XCell II Blot Module) from an in-house 6-10% SDS PAGE gel to a

nitrocellulose membrane (Bio-Rad), membranes were blocked in 5% milk in PBS before being incubated in primary antibody (anti-PAR, clone LP-9610 from BD Biosciences) for 1 hour at room temperature, rinsed in PBS-T, and then incubated in secondary antibody (Anti-Rabbit 800 nm from LI-COR Biosciences) for 1 hour before being imaged on an Odyssey CLx and analyzed in Image Studio (from LI-COR, version 2.0).

SVP digestion of *in vitro* PARylated HisPARP-1

1 μ g of PARylated HisPARP-1 was treated with 500 mUnits of purified SVP in SVP digestion buffer (50 mM Tris pH 7.5, 150 mM NaCl, 15 mM MgCl₂, 20 mM 3-aminobenzamide) for 2 hours at 25 °C, 500rpm.

Testing loss of PARylation by exposure to phosphoelution conditions

hPARP-1 was induced to automodify *in vitro* (as described above), mixed in a 1:2 ratio with BSA and 1 μ g hPARP-1/2 μ g BSA were aliquoted and exposed to either 5% ammonium hydroxide, 500 mM KH₂PO₄ pH 7, or automodification buffer (control) in a total volume of 10 μ L for 5 minutes.

Reactions were quenched by adding 1 mL of ice-cold precipitation buffer (0.02% deoxycholate, 4% Triton X-100, 10% TCA) and stored at -20 °C for 2 hours before being pelleted by centrifugation at 4 °C and decanted. Pellets were washed with ice cold acetone containing 20 μ g/mL glycogen as a carrier, stored at -20 °C for 30 minutes, pelleted, decanted, dried by speedvac and resuspended in SDS Running Buffer. For SDS-PAGE analysis an equal volume of 2x SDS-PAGE buffer was added to samples for analysis on an in-house 6-10% Tris-Glycine gel.

Cell culture

HeLa cells (Kyoto) were grown in arginine and lysine free DMEM (Pierce) containing 10% dialyzed FBS (Sigma), 0.4 mM arginine (¹³C₆¹⁵N₄ from Cambridge, ¹²C₆¹⁴N₄ from Sigma) and 0.8 mM lysine (¹³C₆¹⁵N₂ from Isotec, ¹²C₆¹⁴N₂ from Sigma). Trophoblast stem cells from PARG knockout mice (E3.5 from 129.SVJ mice, acquired from Dr. David Koh of Johns Hopkins University[26]) were

grown in arginine and lysine free RPMI 1640 (Pierce) containing 16% dialyzed FBS (Sigma), 0.4 mM arginine ($^{123}\text{C}_6^{15}\text{N}_4$ from Cambridge, $^{12}\text{C}_6^{14}\text{N}_4$ from Sigma), 0.8 mM lysine ($^{13}\text{C}_6^{15}\text{N}_2$ from Isotec, $^{12}\text{C}_6^{14}\text{N}_2$ from Sigma), 1 mM sodium pyruvate (Life Technologies), 2 mM L-glutamine (Life Technologies), 25 units/mL penicillin (cellgro), 25 units/mL streptomycin (cellgro), 100 uM monothioglycerol (Sigma), 1 $\mu\text{g}/\text{mL}$ heparin sulfate, 25 ng/mL FGF-4 and 0.5 mM benzamide (Sigma). PARC knockout cells were grown without benzamide for 48 hours before harvesting. All cells were treated with 5 mM N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, from AccuStandard) for 5 minutes before being washed three times with ice cold PBS (Gibco) and lysed in either 6M guanidine-hydrochloride (Sigma), 8M urea (Sigma) or lysis buffer (50 mM Tris pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1x EDTA-free cOmplete protease-inhibitor from Roche, 1% NP-40, 1 $\mu\text{g}/\text{mL}$ ADP-HPD and 0.1% sodium deoxycholate). Cells lysed in either guanidine-hydrochloride or urea were subjected to sonication in an ice bath for 10 minutes with 30 second breaks between 30 second cycles (Bioruptor Standard). Cells lysed in lysis buffer were left on ice for 10 minutes. Following lysis all cell debris was cleared by centrifugation.

PAR enrichment by macrodomain

2 mg of whole cell lysate in 1x lysis buffer (50 mM Tris pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1x EDTA-free cOmplete protease-inhibitor from Roche, 1% NP-40, 1 $\mu\text{g}/\text{mL}$ ADP-HPD and 0.1% sodium deoxycholate) were incubated at 5 mg/mL with 40 μL of macrodomain-conjugated agarose beads (Tulip #2302) at 4°C overnight before being washed three times with wash buffer (50 mM Tris pH 7.5, 0.4 M NaCl and 0.1% sodium deoxycholate) and eluted by 8M urea pH 7.

SVP digestion of endogenous proteins with or without protein standard (hPARP-1)

All proteins were denatured in 8M urea pH 7 for 10 minutes at 37°C before being reduced in 1 mM Tris-(2-Carboxyethyl)phosphine (Life Technologies) for 10 minutes and then alkylated in 2 mM 2-chloroacetamide (Sigma) for 10 minutes in the dark. If automodified hPARP-1 is to be added as a standard it is prepared the same way and added into the lysate background at this point. Samples

were then diluted to a final concentration of 1M urea, 50 mM NaCl, 15 mM MgCl₂, 1 mM CaCl₂, and 0.2 M Tris pH 7.3. 5 µg of purified SVP were added for each mg of whole cell lysate and incubated for 2 hours at 37°C.

In-solution protein digestion

Samples in 1 M urea, 0.2 M Tris-Cl pH 7.3, 1 mM CaCl₂, 15 mM MgCl₂, and 50 mM NaCl are treated with endoproteinase LysC (Wako) 1:50 enzyme:substrate. After 1 hour trypsin (Sigma) was added at a 1:50 enzyme:substrate ratio, the entire reaction was incubated overnight. Reaction was stopped by adding an equal volume of desalting solvent A (5% acetonitrile, 0.1% TFA) and desalted on a C18 StageTip and eluted in desalting solvent B (80% acetonitrile, 0.1% TF) as in Rappsilber *et al* [27].

Phosphoenriching peptide standards from HeLa WCL peptide background

HeLa cells were scraped into 6M Gnd-HCl, lysed by sonication and cleared by centrifugation. 300 µg of protein was then reduced, alkylated and in-solution digested by LysC and Trypsin as described in “In-solution protein digestion”. To this mixture of peptides 30 µg of peptides from automodified, SVP-treated hPARP-1 and 10 µg of peptides from bovine casein were added. This mixture was then sampled as input and split into 3 equal volumes which were enriched by either IMAC (Sigma PHOS-Select beads) or MOAC (GL Sciences or GlySci tips containing ZirChrom TiO₂ beads). IMAC samples were enriched as in Villen *et al* 2008[28], briefly: they were incubated for 1 hour, shaking at 25°C, on 50 µL of PHOS-Select beads in binding buffer (0.1% formic acid, 40% acetonitrile). These beads were then transferred to a pre-equilibrated StageTip[27] where they were washed with binding solvent three times, acidified with 1% FA and eluted onto the StageTip with 0.5M potassium phosphate pH 7, where they were acidified with 1% FA again and washed with desalting solvent A (5% acetonitrile, 0.1% TFA). They were then eluted with Desalting Solvent B (80% acetonitrile, 0.1% TFA). MOAC samples were enriched either by GL Sciences or GlySci TiO₂ tips, both by their

manufacturer's protocols with the adaptation that they were eluted with 0.5M potassium phosphate pH 7.

NanoLC-MS/MS analysis

Peptides were separated on a Thermo-Dionex RSLC Nano UPLC instrument with ~10 cm x 75 micron ID fused silica capillary columns with ~10 micron tip opening made in-house with a laser puller (Sutter) and packed with 3 micron reversed phase C18 beads (Reprosil-C18.aq, 120 Angstroms, Dr. Maisch) with a 90 min gradient of 3-35% B at 200 nL/min. Liquid chromatography (LC) solvent A was 0.1% acetic acid and LC solvent B was 0.1% acetic acid, 99.9% acetonitrile. MS data was collected with a Thermo Orbitrap Elite. Data-dependent analysis was applied using Top5 selection and fragmentation was induced by CID and HCD. Profile mode data was collected in all scans.

Database search of MS/MS spectra for peptide & protein identification

Raw files were analyzed by MaxQuant version 1.4.0.8 using protein, peptide and site FDRs of 0.01 and a score minimum of 40 for modified peptides, 0 for unmodified peptides; delta score minimum of 17 for modified peptides, 0 for unmodified peptides. Sequences were searched against the UniProt Human Database (definitions updated May 29th, 2013). Endogenous phospho- and phosphoribose peptide lists were further restricted by a delta ppm of +/- 2σ from each respective data set (average and standard deviation were calculated from the complete tandem mass spectra (MS/MS) list of identified peptide precursors) and the expected heavy/light ratios (greater than or less than 1 for heavy or light data sets, respectively). MaxQuant search parameters: Variable modifications included Oxidation (M), Acetylation (Protein N-term), phosphorylation (STY) and phosphoribosylation (DEKR). Phosphoribosylation (DEKR) allowed for neutral losses of H₃PO₄ (phosphoric acid, 97.98 daltons) and C₅H₉PO₇ (phosphoribose, 212.01 daltons). Carbamidomethyl (C) was a fixed modification. Max labeled amino acids was 3, max missed cleavages was 2, enzyme was Trypsin/P, max charge was 7, multiplicity was 2, SILAC labels were Arg10 and Lys8.

2.4 Results

SVP Treatment of PARylated Substrates Generates Phospho(ribose)ated Proteins, which can be Stained by a Phosphoprotein Dye, Pro-Q Diamond

As a model for protein PARylation we utilized hPARP-1, a Poly(ADP-ribose) Polymerase capable of auto-poly(ADP-ribosyl)ation. Exposure of hPARP-1 to ^{32}P labeled $\beta\text{-NAD}^+$ resulted in an increase in molecular weight of hPARP-1 above its unmodified mass of 113 kDa which was correlated with the ^{32}P signal observed in the autoradiograph, indicating incorporation of ^{32}P -ADPr via hPARP-1 automodification (see Figure 2-1 panels a and b, lane 1 vs. lane 2). Upon treatment with SVP (lane 3), the majority of the “smear” was lost by both Coomassie blue and ^{32}P detection with an accompanied increase in the intensity of the Coomassie-stained band at the expected size of unmodified hPARP-1. This result demonstrates SVP’s ability to break down the polymer at pyrophosphate bonds, potentially reducing the polymer entirely to the phospho-ribosyl group on the modified amino acid residue of PARylated proteins.

Because of the similarity of phospho(ribose) and phosphate groups, we posited that the phospho(ribose)ated hPARP-1 might share properties with phosphoproteins. To test this hypothesis, we used the phosphoprotein gel stain, Pro-Q Diamond, to stain the polyacrylamide gel in Figure 2-1a (Figure 2-1c). While unmodified hPARP-1 and modified hPARP-1 were weakly stained with Pro-Q Diamond, the signal was significantly increased for SVP-treated hPARP-1 (Figure 2-1c, lanes 1-3). The phospho-specificity of the dye was confirmed with the two phosphoprotein controls, ovalbumin and κ -casein, in the protein molecular weight ladder (Figure 2-1c, marker). To confirm that the staining associated with SVP-treated hPARP-1 is due to the presence of phosphate groups, we treated the samples with calf intestinal phosphatase (CIP). As expected, upon removal of the phosphate groups by CIP, the resultant ribosylated hPARP-1 was no longer stained by Pro-Q diamond (Figures 2-1d and 2-1e). These data suggest that SVP treatment of PARylated substrates produces phospho(ribose) groups, and that the resultant phosphate groups may have similar

physicochemical properties to phosphate groups in phosphoproteins. We then sought to examine our ability to enrich these phospho(ribose) groups using phosphopeptide enrichment strategies

Neutral phosphate buffer preserves base-labile ADP-ribose bonds and serves as a safe alternative to ammonia for peptide elution

Popular phosphoproteomic approaches use immobilized metal affinity chromatography (IMAC) or metal oxide affinity chromatography (MOAC) to enrich phosphopeptides, followed by elution with ammonium hydroxide. Unfortunately, ammonium hydroxide is highly basic and therefore releases ADPr from glutamic and aspartic acid residues[13]. For this reason we considered an alternative elution condition, neutral phosphate buffer, which has been used previously to competitively elute phosphopeptides[28]. To assess ADPr stability in the presence of phosphate buffer ^{32}P -labeled, automodified hPARP-1 was exposed to either 5% NH_4OH , 0.5M KH_2PO_4 pH 7 or control (automodification buffer containing 20 mM Tris pH 7.5). As can be seen in Figure 2-2 both the control and the neutral phosphate buffers maintained hPARP-1 in its PARylated form (smeared) while ammonia hydrolyzed PAR, returning much of the hPARP-1 to its native size by Coomassie (Figure 2-2 panel a) and removing ^{32}P -labeled PAR as shown in the autoradiograph (Figure 2-2 panel b). These results suggest that the standard alkaline conditions in phosphoproteomic elution protocols result in the loss of PARylation while the neutral phosphate buffer preserves the ADPr-protein bond and should be a safe method to elute phospho(ribosyl)ated peptides from phospho-affinity matrices.

Quantitative comparison of phosphoproteomic techniques in co-enriching phospho(ribosyl)ated peptides and phosphopeptides

Next, we explored whether phospho(ribosyl)ated peptides can be enriched from cellular complex mixtures using phosphoenrichment matrices. SVP-treated hPARP-1 was mixed with HeLa cell lysate which was SILAC[29] labeled in “heavy” culture medium containing $^{13}\text{C}_6$, $^{15}\text{N}_2$ -Lysine and

$^{13}\text{C}_6, ^{15}\text{N}_4$ -Arginine (Supplementary Figure 2-1). Because we expect the human hPARP-1 spectra to be derived from SVP-treated, unlabeled “light” hPARP-1 samples, we can verify the source of the peptide by SILAC state. As a positive phosphoenrichment control, peptides from known phosphoprotein standards, bovine caseins, were also added to the whole cell lysate background. The complex peptide mixture was subjected to three commercially available phosphoenrichment matrices – (1) Sigma PHOS-Select Iron Affinity Gel (PS), (2) GL Science Titansphere Phos- TiO_2 Tips (GL) and (3) GlySci Phosphopeptide NuTip using Zirchrom Titanium Dioxide beads (ZC). In each case, peptides were eluted with 0.5M potassium phosphate buffer at pH 7.0 to preserve the labile bond between phospho(ribose) and acidic amino acids. Mass spectrometry data was collected on an Orbitrap and fragmentation was induced by both Collision-Induced Dissociation (CID) and Higher-energy C-trap Dissociation (HCD).

Overall, our complex background consisted of 44,655 peptides from 2,148 proteins and included 3,421 endogenous phosphopeptides (see Supplementary Tables 2-2, 2-3, 2-4). Out of this background we identified 47 unique phosphopeptides from the spiked-in phosphoprotein standards (bovine caseins) using all enrichment techniques (Supplementary Table 2-1, Figure 2-3a and Supplementary Figures 2-2a,e). While PHOS-Select contributed the most unique peptide identifications (36%) both GL Sciences and Zirchrom found peptides which would not have otherwise been identified (2% and 13%, respectively). This stands in contrast to the 29 unique hPARP-1 phospho(ribosyl)ated peptides, of which nearly 60% were found exclusively through enrichment by PHOS-Select (Supplementary Table 2-1, Figure 2-3b and Supplementary Figures 2-2b, f) and only a single peptide (3%) was found solely by an alternative enrichment (Zirchrom). Further assessment of the PHOS-Select enrichment profile reveals that the 39 unique phosphopeptides and 28 unique phospho(ribose) peptides found in the PHOS-Select eluate entirely overlapped with the small number of peptides which were found in the respective input and flowthrough analyses (Figures 2-3c,d and Supplementary Figures 2-2c,d,g,h).

In order to determine whether the protocol proposed here is as robust for phospho(ribosyl)ated peptides as phosphopeptides a serial enrichment was performed which included re-enriching the flowthrough sample multiple times to quantify the depletion of these two classes of target peptides (figure 2-4). Automodified hPARP-1 was again used as the PAR standard however this time the PARylated hPARP-1 was denatured in 8M urea, reduced and alkylated prior to being added in to the whole cell lysate background (figure 2-4a). This denaturation step served to completely inactivate hPARP-1 (see supplementary figure 2-3) thus allowing us to perform SVP digestion of the whole cell lysate and the PARylated standard in the same mixture. Furthermore the His-tag on hPARP-1 allowed us to isolate a portion of the standard back out of the mixture both before and after SVP digestion, these samples served as a quality control step as the loss of PAR and the formation of phospho(ribose) could be monitored by SDS-PAGE and western blot (see figures 2-4 panels b-d). As shown in figure 2-4e both classes of peptides are depleted from the background population at similar rates (as opposed to phosphopeptides being enriched preferentially prior to phospho(ribosyl)ated peptides), indicating that the IMAC method proposed is truly a dual enrichment of both phospho- and phospho(ribosyl)ated peptides. It should be noted that the peptides identified in this study include both those from the hPARP-1 standard as well as the endogenous phospho- and phospho(ribosyl)ation sites from the MNNG-treated murine PARG knockout cells used to generate the heavy-labeled complex background. For a complete list of endogenous phospho- and phospho(ribosyl)ated peptides see supplementary tables 2-5 and 2-6.

Characteristics of Phospho(ribosyl)ated peptides

Among the phospho(ribosyl)ated peptides identified from the hPARP-1 standard, 20 unique sites were modified. Many of these sites were outside of the automodification/BRCT domains that are known to be heavily PARylated[30] (Table 2-1), and in fact, over one-third of the sites identified (7/20) are in the second zinc finger which is not strictly required for PARP-1 activation [31]. Of the 20 potential PARylation sites 1 arginine, 3 lysine, 4 aspartate and 12 glutamate residues were identified. While the basic sites may seem surprising we emphasize that the inherent NADase activity

of PARP-1 [32] has the potential to create free ADP-ribose, a molecule which can spontaneously modify basic sites independent of PARP-1's conjugation activity [33]. As this non-enzymatic mechanism of ADP-ribosylation is still under investigation we believe the ability of this method to identify the presence of ADP-ribose on both basic and acidic modifications will prove highly useful in elucidating methods of ADP-ribose modification and automodification.

Among the 20 hPARP-1 automodification sites identified 10 were independently verified as endogenous sites in DNA damaged cells in a recent analysis [14]. While most peptides presented with a single phospho(ribose) there were 3 examples of doubly phospho(ribosyl)ated peptides which demonstrated the ability of hPARP-1 to place these large, negatively charged polymers within close proximity of each other (Supplementary Spectra). A notable example of this is the dual modification of E488 and E491, PARP-1 automodification sites which have been independently verified by a number of groups, including Zhang *et al* who identified them as endogenous ADP-ribosylation sites (Table 2-1). Here, we have shown the fragmentation patterns of the unmodified, singly modified and doubly modified forms of this peptide by both CID (Figure 2-5) and HCD (Supplementary Figure 2-4) indicating the shift in molecular weight corresponding to a single (circle) or a double (square) phospho(ribose) group. The doubly-modified peptide also demonstrates the potential for neutral loss of phosphoric acid (H_3PO_4 97.98 daltons) and phospho(ribose) ($\text{C}_5\text{H}_9\text{PO}_7$, 212.01 daltons) from the parent ion upon fragmentation (Figure 2-5, Supplementary Figure 2-5) - these neutral losses were observed in 73% (16/22) of the spectra annotated for validation of the PARP-1 automodification sites (Supplementary Spectra), most often showing up in the presence of the modified form, indicating that neutral loss was not complete. Considering how common these neutral losses are the authors advise including them in mass spectrometry search criteria.

Our analysis identified three lysine modifications: two novel, one previously reported in a 2009 mutagenesis screen[16] (Table 2-1). Two of these were found at the C-terminus of the peptide, suggesting that the phospho(ribosyl)ated residue did not prevent proteolytic cleavage at the modified lysine, in our case by a combination of LysC and Trypsin. To confidently assign the novel

PARylation site K486, its CID fragmentation pattern was compared to an unmodified version of the same peptide – revealing a b-ion series which was unmodified in both spectra and a y-ion series which contained the 212.01 Da shift indicative of a phospho(ribose) addition to every y-ion fragment (figure 2-6). The extensive b- and y-ion series provide strong evidence of the phospho(ribose) modification on the peptide C-terminal lysine, demonstrating (1) the availability of phospho(ribosyl)ated lysines for protease cleavage and (2) the ability of PHOS-Select to enrich phospho(ribosyl)ated lysines.

ADP-ribosylation sites identified from whole cells

In order to establish a pipeline for identifying endogenous sites of mono and poly(ADP-ribosylation) HeLa cells were SILAC labeled and then treated with the DNA damaging agent MNNG to induce PARylation before being subjected to an affinity pull-down by the mono and poly(ADP-ribose) binding macrodomain from A/1521[34]. ADP-ribosylated proteins were then denatured before being treated with SVP and then digested with a mixture of the proteases LysC and trypsin. These peptide mixtures were then split in half and either enriched over a charged or a stripped IMAC resin with the elution from the stripped resin serving as a nonspecific background control for the eluted peptides which had come off of the charged IMAC resin (see figure 2-7a). Since both forward and reverse labeling patterns were used the strongest hits from the database showed up in both populations, as demonstrated in figures 2-7b and 2-7c. A representative spectrum for phosphoribosylated R4 from Serine/Arginine rich Splicing Factor 2 (SRSF2) is shown with its parent ion in figures 2-7b and 2-7d. Notably, the pipeline described in 2-7a was performed in parallel on an MNNG-treated trophoblast stem cell line from a PARG knockout mouse model [26], producing 22 unique endogenous phospho(ribosyl)ated peptides, two of which (containing R4 from SRSF2 and R199 from heterogeneous nuclear ribonucleoprotein U, HNRNPU) overlapped with those found from the HeLa preparation (see supplementary table 2-5). All of the phospho(ribosyl)ated peptides identified from these samples were found in the IMAC enriched

fractions, indicating that macrodomain enrichment followed by SVP digestion was not sufficient for site identification.

In order to determine whether the macrodomain enrichment was necessary for site identification the same analysis was done with the ADPr affinity purification omitted, again utilizing both the human wildtype and murine PARG knockout cell lines described above. 22 unique phospho(ribosyl)ated peptides were identified from these preparations, including the same HNRNPU peptide containing R199 found following macrodomain enrichment (it was again found in both cell lines), showing that the macrodomain enrichment is not only insufficient on its own for site identification but also that it is not necessary. Furthermore, a comparison of the macrodomain enriched versus unenriched data sets revealed a bias in the amino acids which served as attachment sites for phospho(ribose); the macrodomain enrichment appears to have shifted the profile of ADP-ribosylated amino acids away from glutamic acid residues (figure 2-8, source data can be found in the supplementary text and supplementary table 2-5). This shift indicates that the macrodomain is either selecting against ADP-ribosylated glutamic acid in favor of other amino acid attachment sites or that it is actually removing the ADP-ribose attachment from glutamic acids. The latter hypothesis lines up with recently published work showing that the macrodomain of Afl521 possesses ADP-ribosylhydrolase activity, and suggests that this activity may be targeted toward glutamic acid sites of ADP-ribosylation [35, 36].

2.5 Discussion

The expanding relevance of PARylation in cellular processes has led researchers to look beyond the canonical role of DNA repair when considering the consequences of altered PARylation levels [37]. To this end, the most powerful tool for studying global changes in post-translational modifications continues to be systematic analyses of proteomes by mass spectrometry. Unfortunately the widespread use of proteomics and mass spectrometry has not yet been established in the field of PARylation due to challenges relating both to the modification itself – which may be labile, large, and highly charged – and to the low levels of PARylation which exist below the threshold of most

analytical techniques. In response, enrichment techniques have been developed which have allowed researchers to study the PARylated proteome with the caveat that the identified proteins are either PAR acceptors or PAR binders; due to the lack of site identification in these studies verification of which class these proteins belong to is both tedious and, in some cases, impossible [38, 39]. Recently a study has demonstrated the feasibility of identifying mono and poly(ADP-ribosylation) sites in a large proteomic screen which combines enrichment of ADP-ribosylated substrates by boronate chromatography with the removal of ADPr from substrates by hydroxylamine; this chemical treatment allows for subsequent identification of acidic ADP-ribosylation sites by the diagnostic 15.01 Da hydroxamic acid derivative left behind [14]. The limitation in this study was that ADP-ribosylated lysine and arginine could not be detected as only acidic residues were left with the hydroxamic acid tag. In contrast, our pipeline can identify ADP-ribosylation attachment sites on both acidic and basic residues; it should also be noted that this universality allows for the discovery of novel amino acid attachment sites for ADPr beyond these acids and bases, the existence of which has not been ruled out. We believe our proposed method of enriching and identifying ADP-ribosylation sites addresses the need for a pipeline which is both global and definitive in identifying ADPr acceptors at the protein and amino acid levels.

The phosphoenrichment methods applied in this study have gained popularity in the phosphoproteomic field due to their high specificity and compatibility with both MALDI and ESI LC/MS. MOAC, or Metal Oxide Affinity Chromatography, has proven to enrich phosphopeptides more specifically than its predecessor, IMAC (Immobilized Metal Affinity Chromatography) likely due to the tighter binding of phosphate to the TiO_2 microspheres (titanospheres) as compared to the chelated iron used by PHOS-Select IMAC resin [40]. This tight binding, however, may explain the lack of phospho- and phospho(ribose) peptides found in the eluates from the TiO_2 resins used here (GL Sciences and ZirChrom) which have an optimal elution pH between 9.2 and 9.4 [41]. IMAC elution, on the other hand, is much more sensitive to competitive phosphate levels than it is to pH, and does not have an optimal elution pH [42]. We have demonstrated the stability of ADPr protein

attachment sites in neutral phosphate buffer as compared to basic NH_4OH and have restricted our elution conditions to ensure retention of phospho(ribose) on target peptides throughout the enrichment. This consideration may have favored the lower-affinity phosphoenrichment matrix, allowing for a single enrichment protocol capable of enriching both phospho- and phospho(ribose) peptides, perhaps at the expense of tightly-bound phosphopeptides left on the TiO_2 matrices. For thorough phosphopeptide analysis it may be prudent to perform a parallel enrichment with optimal (ie basic) elution conditions from a TiO_2 matrix.

While validating the presence of our phospho(ribosyl)ated protein sample we discovered that the phosphoprotein SDS-PAGE gel stain, Pro-Q Diamond, can act as an indicator of phospho(ribose) modified proteins. While we did not do any in-gel digests, the compatibility of Pro-Q Diamond with downstream LC/MS analysis [43] suggests that isolation and identification of phospho(ribosyl)ated proteins, as well as their PAR acceptor sites, may be possible for researchers who wish to analyze changes in SDS-PAGE profiles. We believe this data-dependent approach would greatly complement the global analysis already offered by the phospho(ribose) ADP-ribosylation tag.

Optimization of our phosphoenrichment protocol presented us with a database of spectra identifying phospho(ribosyl)ated peptides from automodified hPARP-1, ultimately yielding 20 modified sites, eight of which are being reported for the first time. These spectra afforded us the opportunity to characterize phospho(ribosyl)ated peptides (and by extrapolation, ADP-ribosylation sites) with regard to their identification by CID- and HCD-assisted LC/MS/MS. Firstly, we determined that multiple PARylation sites may exist within the same peptide, suggesting hPARP-1 is capable of placing these large, highly charged polymers within an amino acid of each other (as in the hPARP-1 automodified peptide GFSLLATE*D*K, see Supplementary Spectra). The steric hindrance and charge-repulsion associated with neighboring PARylation sites may require a high level of flexibility from the protein, poly(ADP-ribose), or both. Secondly, it is worth noting that we identified two lysine hPARP-1 automodification sites at the C-terminal end of their respective

peptides, indicating that these modified lysines were available for proteolytic digestion (see Supplementary Table 2-2). Finally, fragmentation by HCD and CID revealed the potential of phospho(ribose) to be partially or fully lost in the form of a phosphoric acid or phosphoribose, respectively (see Supplementary Figure 2-5). This loss is not complete as the fragments portraying the neutral loss are often accompanied by otherwise-identical fragments which have maintained the full modification. In the future these neutral loss fragments may serve as diagnostic indicators of peptide phospho(ribosyl)ation state. Recognition of these attributes will aid in the analysis of large, phospho(ribosyl)ated proteomes, which may present these characteristics that would allow them to be ignored by erroneous search parameters.

While demonstrating the application of this method to identify ADP-ribosylation sites from whole cells we validated several known sites of ADP-ribosylation recently identified by a complementary mass spectrometry approach [14] as well as a host of novel sites on both novel and known acceptors of mono and/or poly(ADP-ribose) (see supplementary table 5). One of our most interesting hits lies on K350 of heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1), a protein which was first shown to be poly(ADP-ribosyl)ated in whole cells in 1982 and 12 years later was shown to be one of the 2 major acceptors of ADP-ribose in HeLa cells [44, 45]. More recently, PARylation of HNRNPA1 has been shown to affect splicing, stem cell maintenance and oocyte localization in drosophila, suggesting an interesting role for mammalian HNRNPA1 PARylation [46, 47]. While there have been several proteomic studies which have identified HNRNPA1 in poly or mono(ADP-ribosyl)ation purification schemes [38, 39] this finding is the first indication of the site of PARylation on HNRNPA1 (spectrum annotated in supplementary figure 2-6).

In summary we have proposed and demonstrated the feasibility of a global, unbiased approach for characterizing the mono and poly(ADP-ribosyl)ated proteome. Our technique, based on the digestion of ADPr down to its phospho(ribose) attachment site, allows for enrichment at the peptide level of both acidic and basic ADPr acceptor sites. Furthermore we have shown that our

method allows researchers to find sites of ADP-ribosylation without having to knockdown ADP-ribose hydrolases or perform an enrichment of the ADP-ribosylated proteome, steps which may otherwise introduce bias. Finally, this approach presents a unique opportunity to study the changes in the ADP-ribosylated proteome alongside the co-enriched phosphoproteome. It is our hope that the accessibility of the techniques employed in this enrichment pipeline will allow researchers to characterize global ADP-ribosylation at the level of the amino acid, ultimately resulting in a greater understanding of both mono and poly(ADP-ribose) function and regulation from the bottom up.

2.6 Acknowledgements

The authors would like to thank Dr. John Pascal for providing the hPARP-1 expression plasmid used to synthesize all hPARP-1 protein used here [24], as well as Dr. David Koh who provided the PARG knockout cells used for endogenous ADP-ribose site identification [26]. The research was funded by a Department of Defense Breast Cancer Research Program Idea Award #BC101881 (A.K.L.), a Journal of Cell Science Travelling Fellowship (C.M.D), an NCI training grant 5T32CA009110-35 (C.M.D) and NIDA P30DA028846-01 (S-E.O).

2.7 Figures

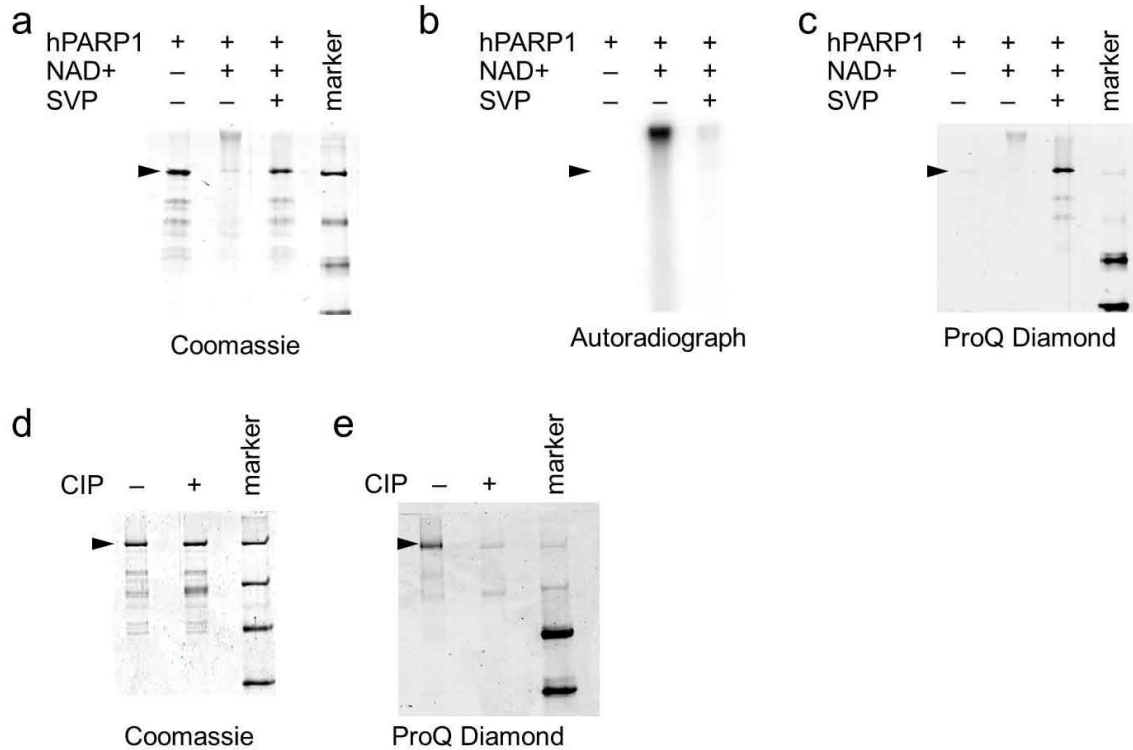


Figure 2-1 Visualizing phospho(ribose) tags on hPARP-1.

(a-c) hPARP-1 automodified *in vitro* upon exposure to ^{32}P -labeled NAD⁺, PAR formation is evidenced by the ^{32}P -labeled smear above unmodified hPARP-1 (arrowheads). Upon treatment with SVP the smear diminishes while the native sized hPARP-1 band reappears. Staining with the phosphostain Pro-Q Diamond indicates that this band is carrying phospho-groups, likely phosphoribose. (d-e) The Pro-Q positive product of SVP-treated automodified hPARP-1 is susceptible to calf intestinal phosphatase (CIP) treatment.

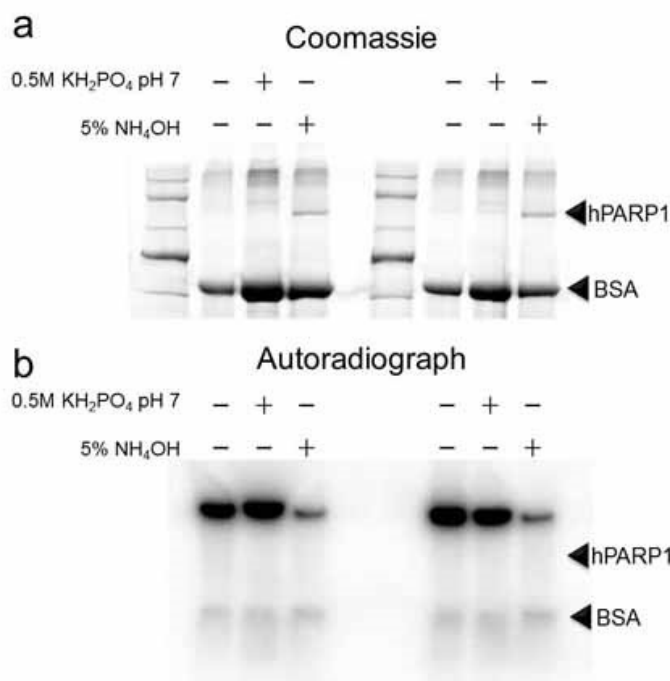


Figure 2-2 Poly(ADP-ribose) is stable in the presence of neutral phosphate buffer.

(a) Coomassie staining shows that PARylated hPARP-1 returns to its unmodified size upon treatment with ammonia for 5 minutes, while neutral phosphate retains the PARylation state as well as the control buffer (automodification buffer). (b) ³²P-labeled PAR shows that the loss of PAR is correlated with the return of hPARP-1 to its native size. BSA (Bovine Serum Albumin) was included as a carrier for sample cleanup by protein precipitation, which was the method applied to immediately quench the chemical exposure.

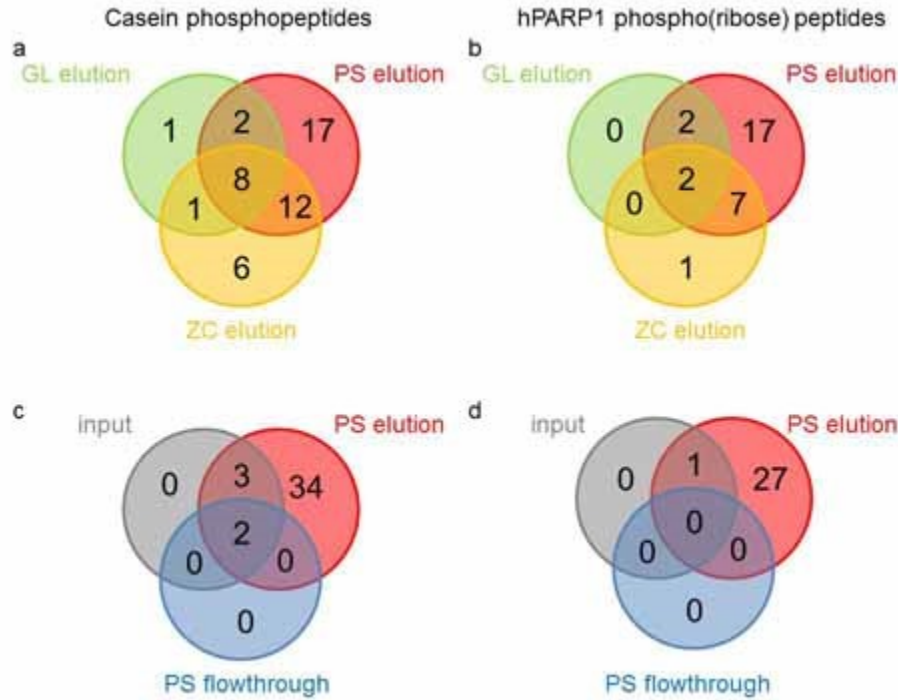


Figure 2-3 IMAC and MOAC enrichment of phospho- & phospho(ribose) peptides.

IMAC (PHOS-Select, PS) was compared to MOAC (both ZirChrom, ZC and GL Sciences, GL) for enrichment of phosphopeptides (from bovine casein) and phospho(ribose) peptides (from hPARP-1) out of HeLa whole cell lysate background. **(a,b)** Unique phosphorylated (a) and phospho(ribosyl)ated (b) peptides identified in eluates from the 3 methods. **(c,d)** Unique phosphorylated (c) and phospho(ribosyl)ated (d) peptides identified in the unenriched (input), elution, and flowthrough from the IMAC method.

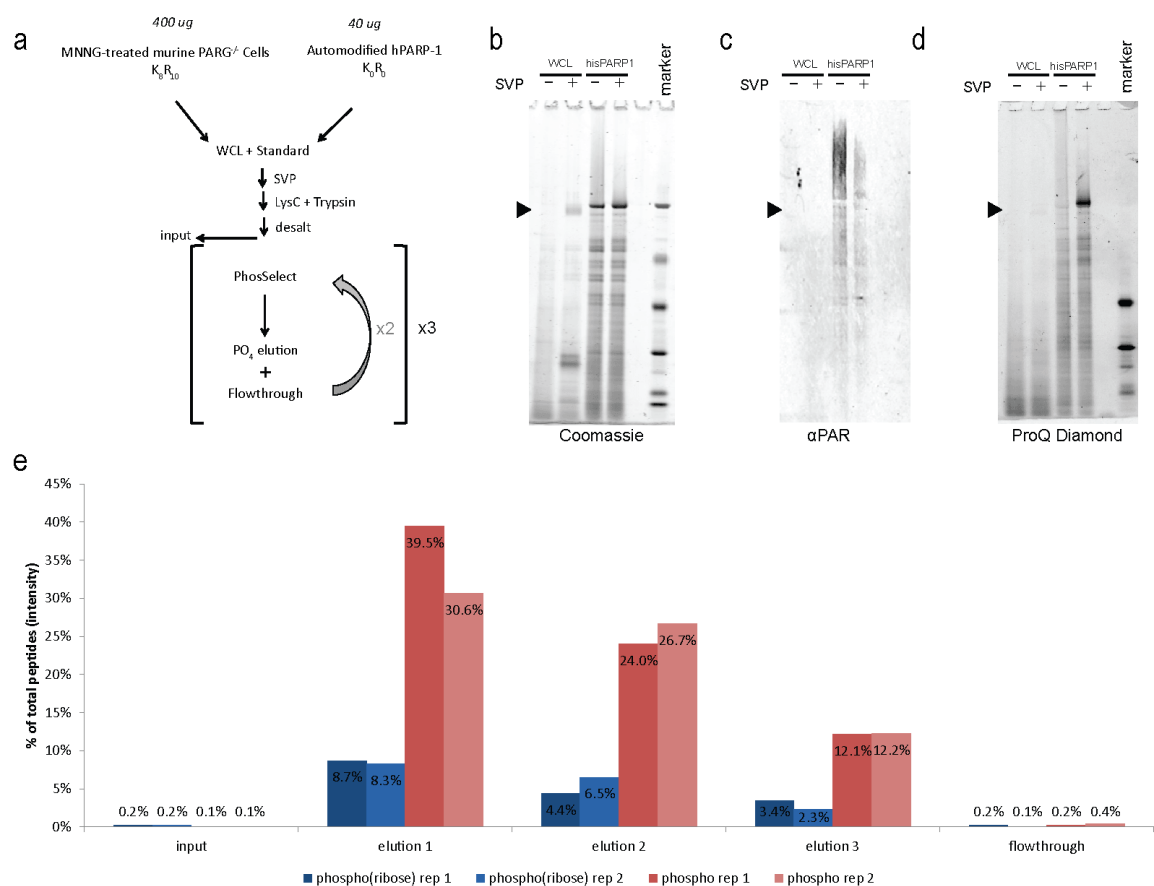


Figure 2-4 Serial enrichment of phospho- and phospho(ribose)lated peptides out of a complex mixture.

His-tagged, automodified PARP-1 was denatured in 8M urea and spiked into heavy-labeled whole cell lysate from MNNG-treated murine PARG knockout cells before being treated by SVP and then digested to peptides and enriched three times in a row on IMAC beads (a). Samples were taken before and after SVP treatment and the His-tagged PARP-1 was separated from the whole cell lysate by nickel IP, allowing visualization of the total protein (b), PARylated his-PARP-1 (c), and phospho(ribose)lated his-PARP-1 (d). MS/MS analysis of the serial enrichments showed that the endogenous phospho-peptides and the phospho(ribose)lated PARP-1 peptides were depleted from the complex mixture at similar rates (e).

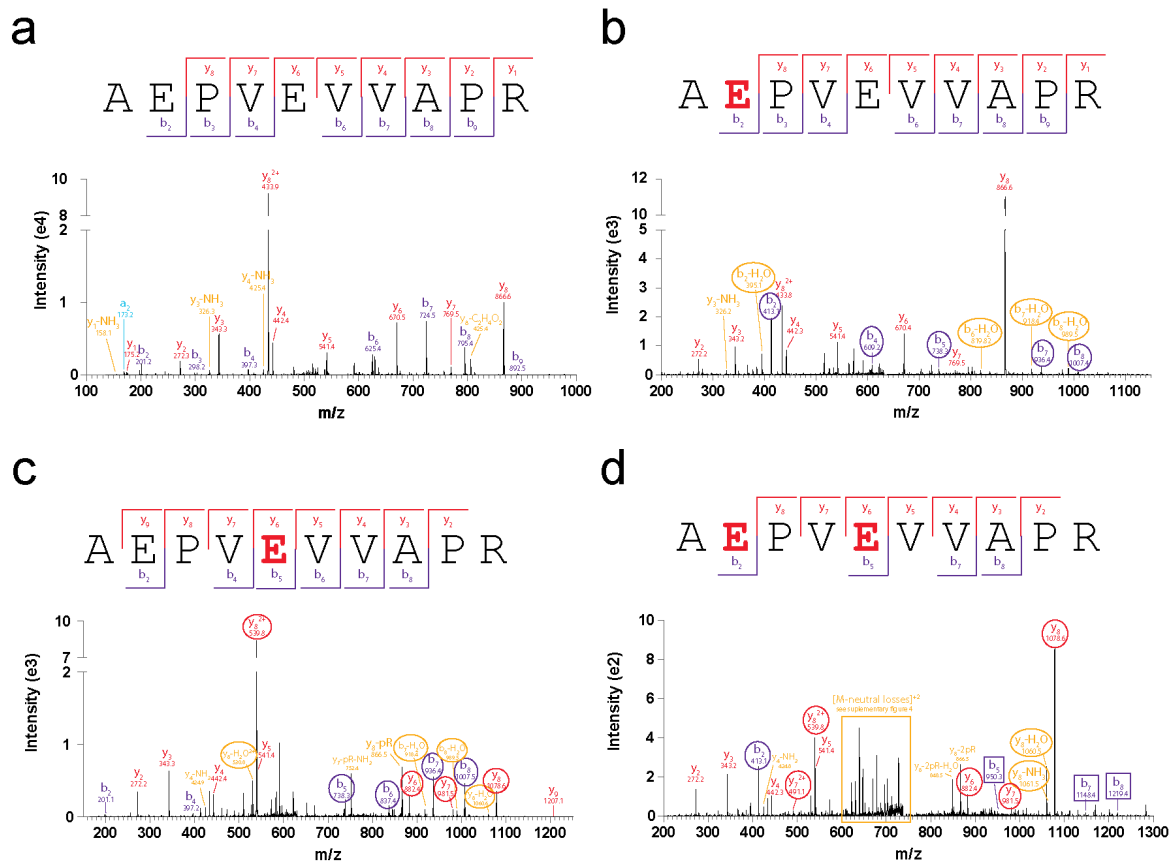


Figure 2-5 Proximal phospho(ribosyl)ation sites.

E488 and E491 are previously characterized PARP-1 automodification sites, shown here in (b) and (c), respectively, as compared to the unmodified form of the peptide shown in (a). The doubly modified peptide (d) contain diagnostic fragments which carry 1 phosphoribose group (212.01 Daltons, circles) as well as those carrying 2 phosphoribose groups (424.02 Daltons, squares).

AA	#	Domain	Novel	endogenous ^x
E	76	ZF1	N ^x	Y
D	112	ZF2	Y	
E	116		N ^{°x}	Y
D	145		Y	
E	147		N ^{Λ°}	
E	168		N ^{Λx}	Y
E	190		N ^{Λ°x}	Y
D	191		Y	
K	239	ZF3	Y	
R	452	BRCT	Y	
E	471		N ^{Λx}	Y
E	484		N ^{Λx}	Y
K	486		Y	
E	488		N ^{*Λ°x}	Y
E	491		N ^{*Λ°x}	Y
K	498	undefined	N [°]	
E	619	WGR	Y	
E	642		N ^x	Y
D	648		N ^x	Y
E	649		Y	

Table 2-1 PARP-1 automodification sites identified.

A total of 20 automodification sites were identified on the PARP-1 standard used for assessing phosphoenrichment techniques. 12 of these sites were previously identified and are annotated as such here: [°]Altmeyer *et al*, 2009[16], ^{*}Tao *et al*, 2009 [48], [^]Sharifi *et al*, 2013 [49], [°]Chapman *et al*, 2013 [23], ^xZhang *et al*, 2013 [14]. Those which were identified by Zhang et al are known to be endogenous PARylation sites. ZF1 = Zinc Finger 1, ZF2 = Zinc Finger 2, BRCT = BRCA1 C-terminus, WGR = tryptophan, glycine, arginine-rich

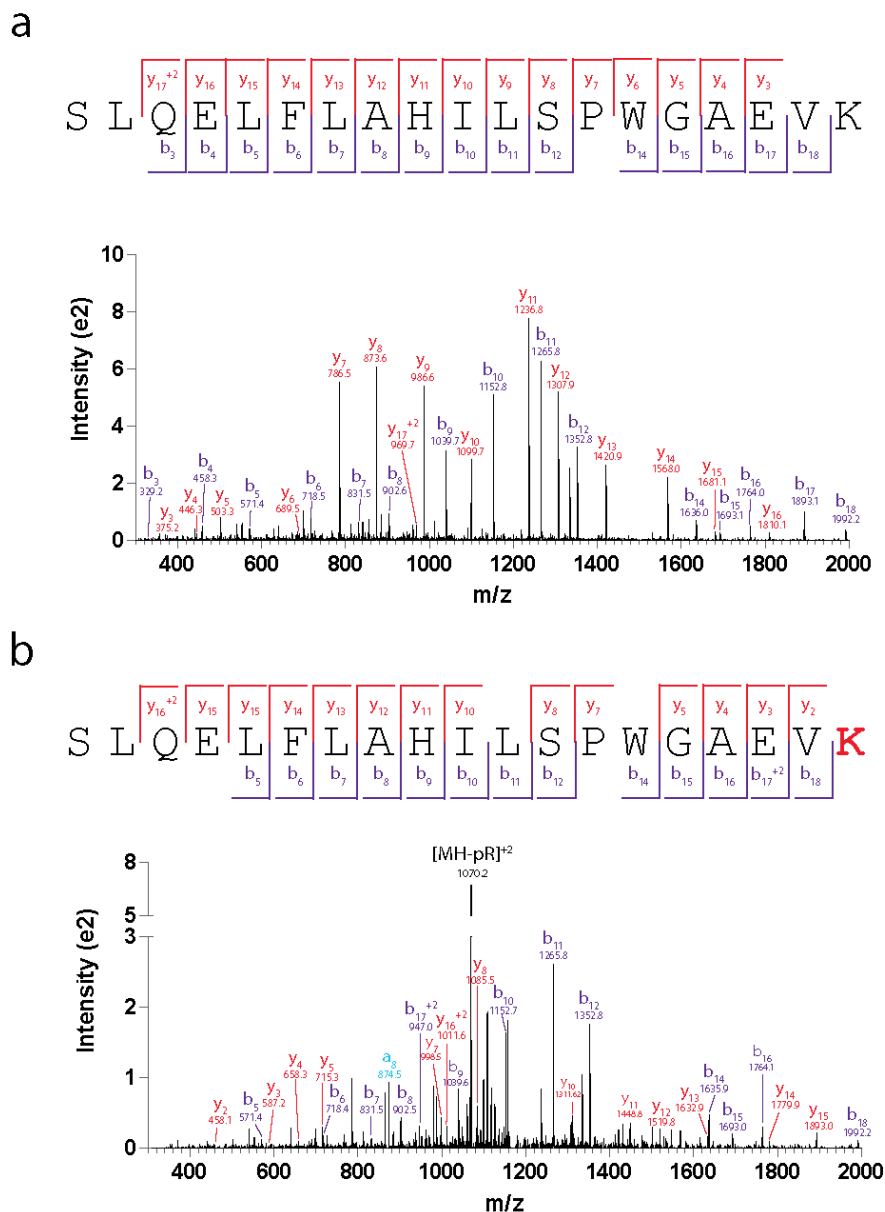


Figure 2-6 Phospho(ribose)lation on peptide terminal lysine.

K486, a novel PARP-1 PARylation site identified in our analysis, is shown here at the peptide C-terminus (b). This fragmentation pattern is compared to that of the unmodified form (a) showing the characteristic 212.01 Dalton shift present in the entire y-series, but absent from the b-series, validating the localization of phospho(ribose).

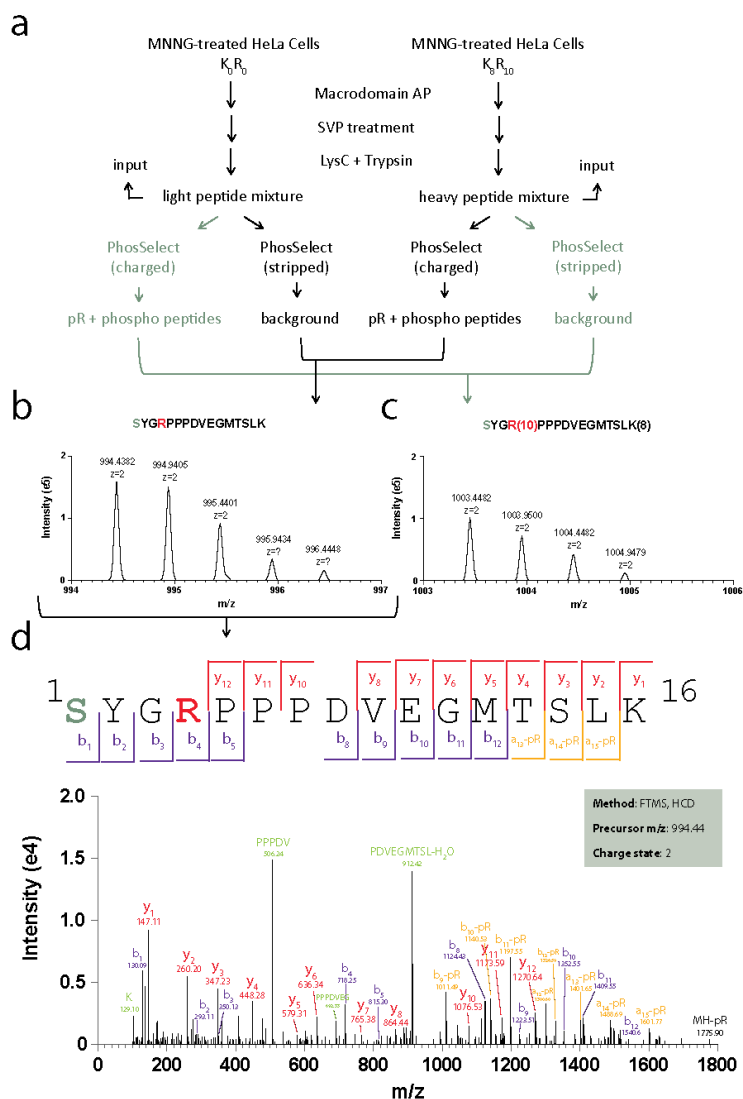


Figure 2-7 Endogenous ADP-ribosylation of Arginine.

In order to identify ADP-ribosylation sites from whole cells we MNNG treated HeLa cells which were either heavy (K_8R_{10}) or light (K_0R_0) labeled, affinity enriched ADP-ribosylated proteins, treated these proteins with SVP to yield phosphoribose, and then digested these proteins to a peptide mixture which would then be enriched either by charged or stripped IMAC beads (a). Stripped IMAC beads from each population would serve as a background control for the reverse labeled peptides enriched over a charged matrix. This example shows the MS (b, c) spectra of both the heavy and light form of R4 from Serine/Arginine Rich Splicing Factor 2 (SRSF2), as well as the annotated MS/MS of the light form (d).

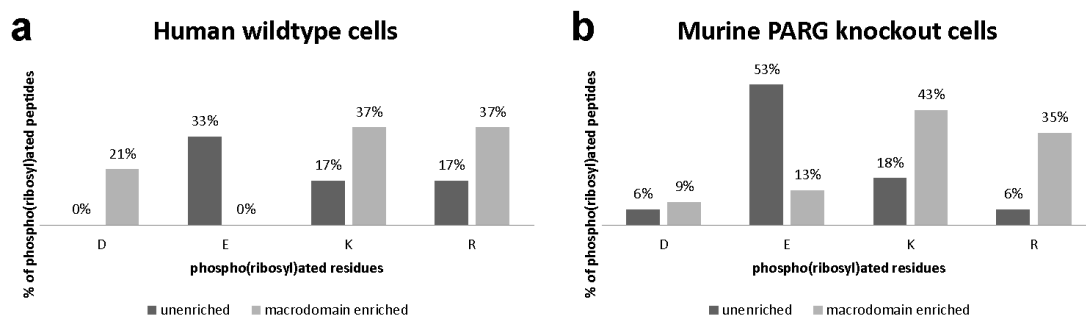
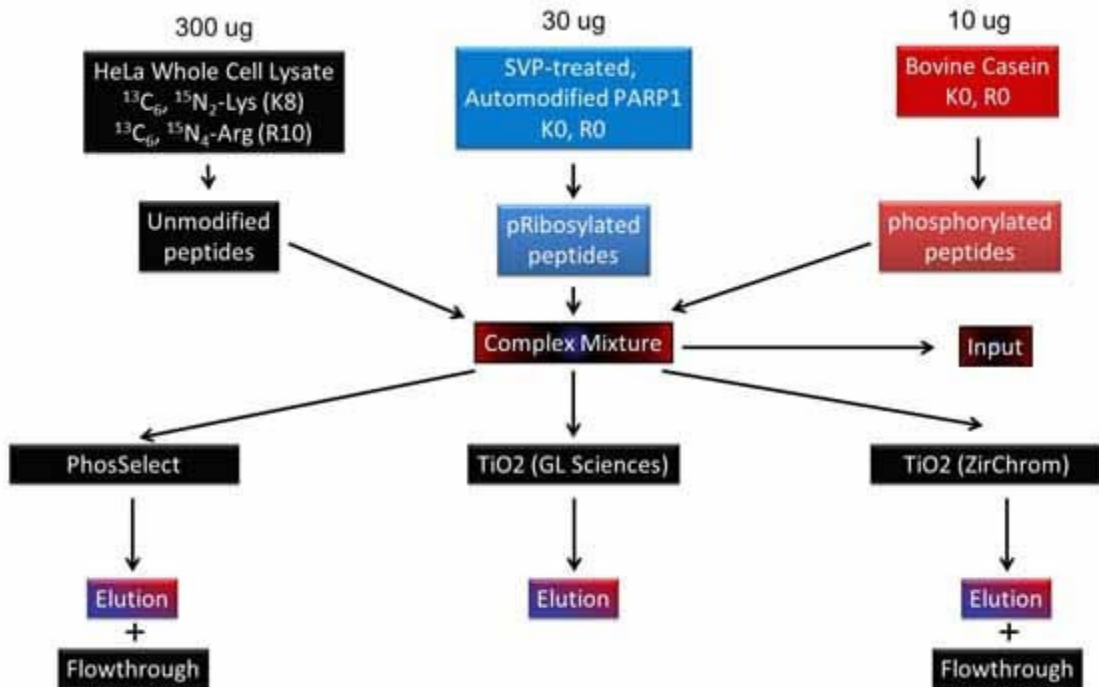


Figure 2-8 The effect of macrodomain enrichment on the (ADP-ribose)lated amino acid profile.

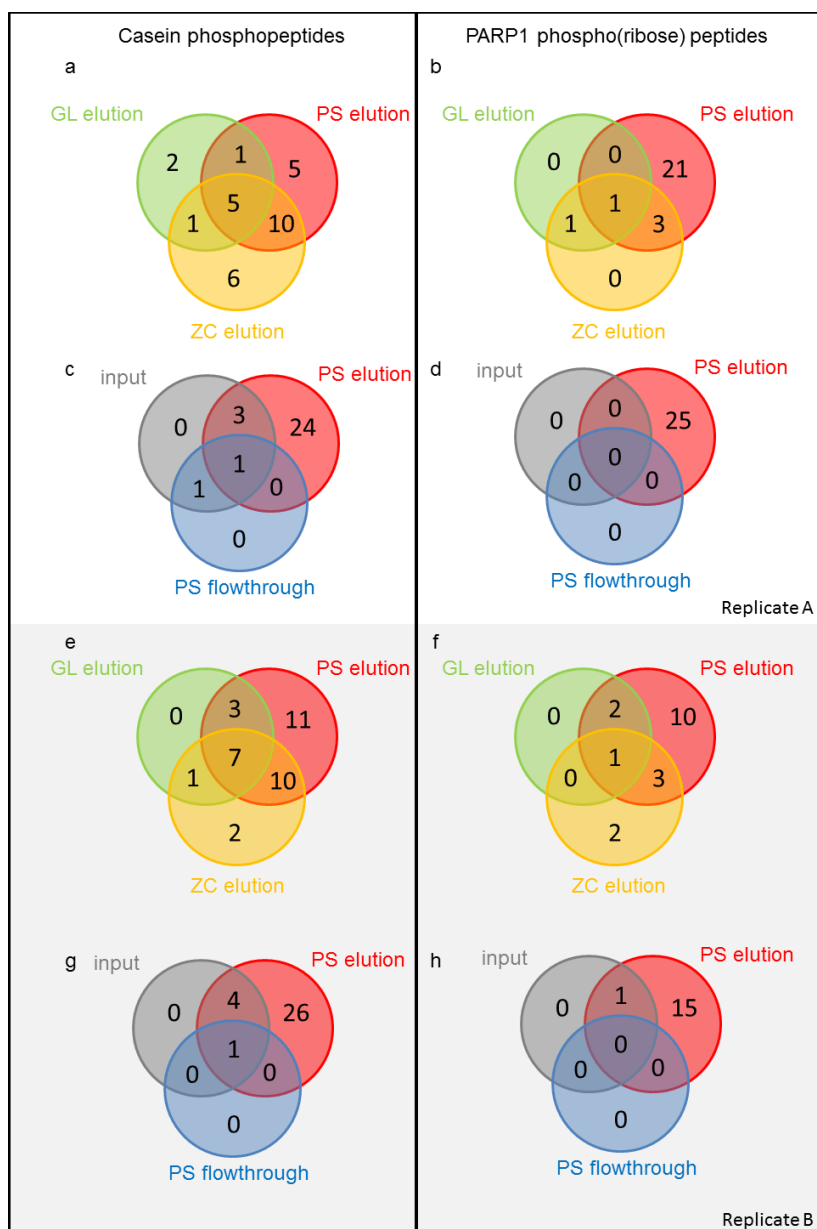
Unique phospho(ribose)lated peptides identified from whole cells, as detailed in supplementary table 2-5, show a shift in the profile of amino acids carrying phospho(ribose) from both human wildtype (a) and murine PARG knockout cells (b).

2.8 Supplementary Figures



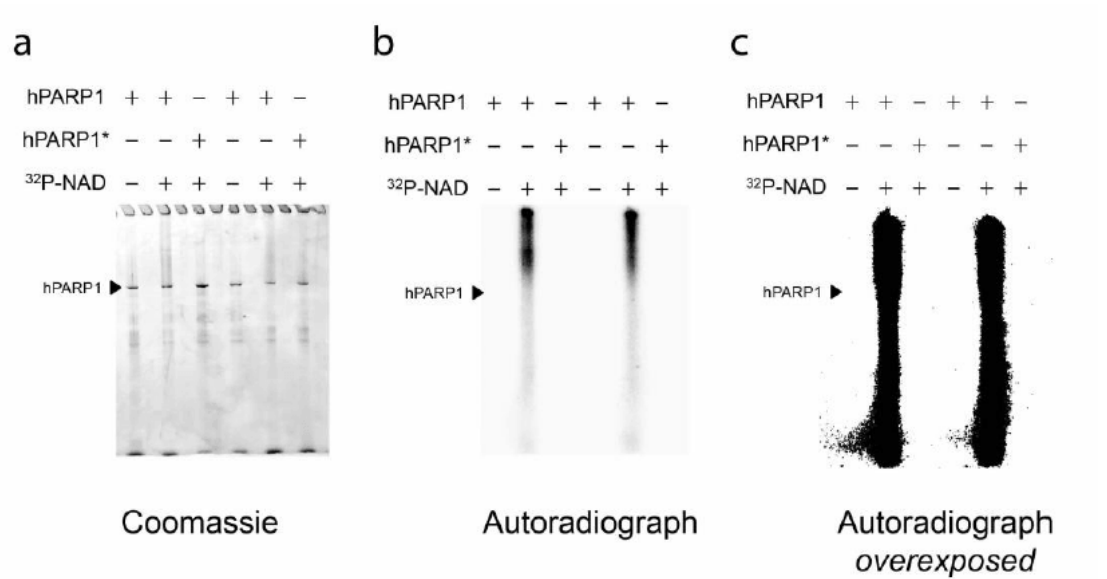
Supplementary Figure 2-1 Enriching phospho- and phospho(ribose) peptides from a complex background

Phospho- and phospho(ribose) peptides were produced from Bovine Casein and SVP-treated automodified hPARP-1, respectively. Concurrently heavy-labeled HeLa whole cell lysate was digested to generate a background of mostly unmodified peptides from which the standards were mixed into and then enriched by either PHOS-Select, GL Sciences or ZirChrom matrices. All digestions utilized both LysC and Trypsin proteases.



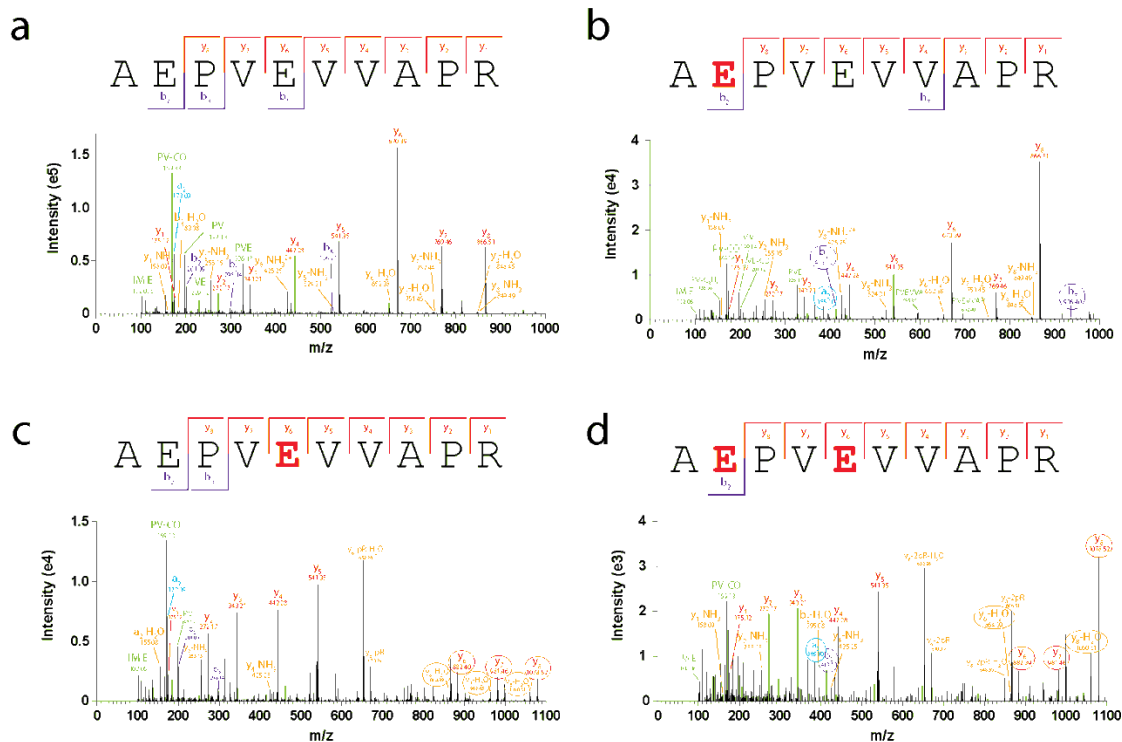
Supplementary Figure 2-2 Co-enriching phospho(ribose)ated peptides by IMAC and MOAC.

IMAC (PHOS-Select, PS) was compared to MOAC (GL Sciences, GL and ZirChrom, ZC) for enrichment of phospho- and phospho(ribose) peptides utilizing a neutral phosphate elution buffer, experiment performed in duplicate (Replicates A & B). Unique peptides from each eluate were compared (a,b,e,f) and unique peptides from the most promising technique, IMAC (PS), were compared to those from the input (unenriched) and flowthrough samples (c,d,g,h). Peptide details can be found in Supplementary Table 2-1 and combined data sets are shown in Figure 2-3.



Supplementary Figure 2-3 PARP-1 is inactivated upon exposure to 8M urea.

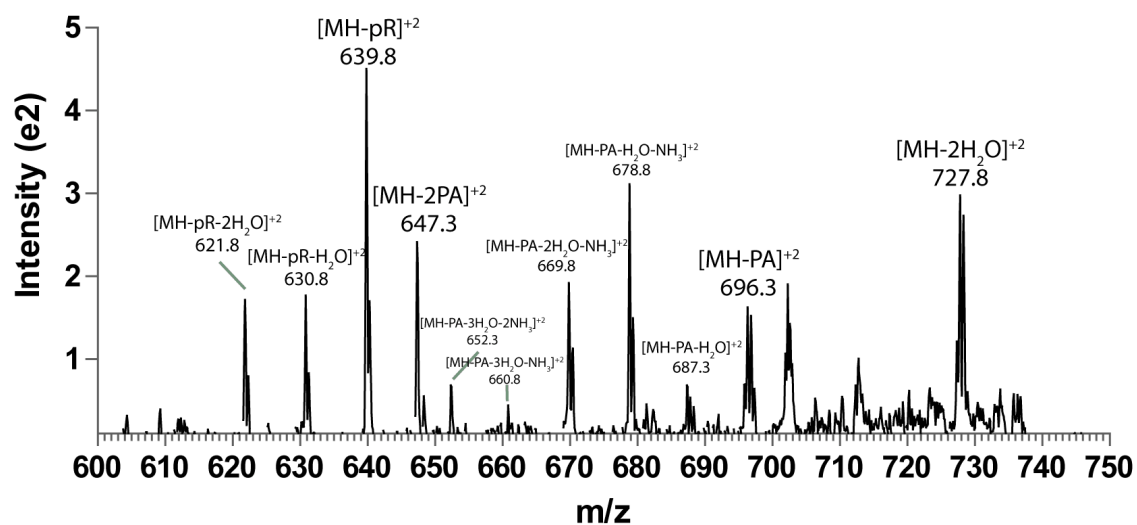
The experimental design depicted in supplementary figure 2-3 shows that the poly(ADP-ribosyl)ated standard hPARP-1 is spiked into whole cell lysate following denaturation by 8M urea. Here we have shown that denatured hPARP-1 (hPARP-1*) has no poly(ADP-ribosyl)ation activity as shown by a failure to automodify in the presence of ³²P-NAD. Native sized-unmodified PARP-1 is shown in (a) as a band around 120 kD, poly(ADP-ribose) is shown as a smear on the autoradiograph (b) which has been overexposed to show a complete failure of hPARP-1* to automodify (c).



Supplementary Figure 2-4 Multiple PAR sites can exist on the same peptide.

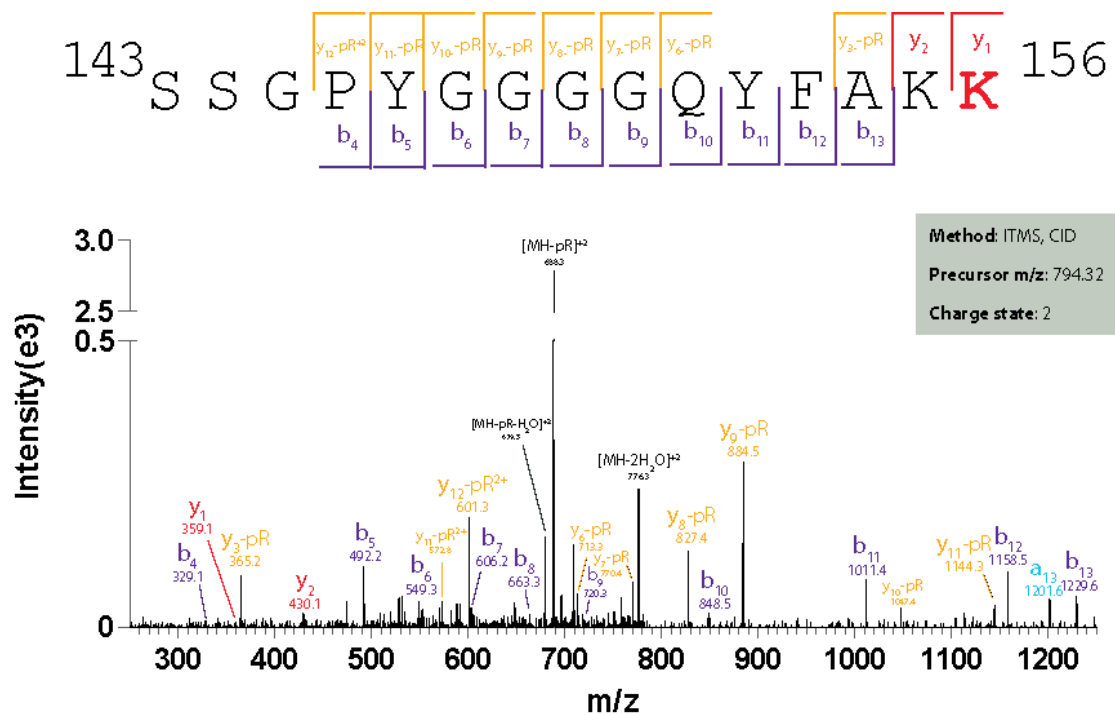
E488 and E491 are known PARP-1 automodification sites – here they are shown with HCD fragmentation (b,c) as compared to the unmodified form (a). Ions which have shifted from the unmodified form by the characteristic 212.01 Daltons of phospho(ribose) are marked with a circle. The doubly-modified form of the peptide, shown in (d), contains ions seen in the unmodified and both singly modified forms, indicating that the peptide is modified at both E488 and E491 residues.

A **E** P V **E** V V A P R



Supplementary Figure 2-5 Neutral loss sequence from a doubly-modified hPARP-1 peptide.

This blow-up of the 600-750 m/z window found in Figure 2-4 panel d reveals a neutral loss sequence based on the dual phospho(ribose) modifications. pR = phosphoribose, PA = phosphoric acid.



Supplementary Figure 2-6 K350 of HNRNPA1 is phospho(ribosyl)ated.

Site assignment of the phospho(ribosyl)ated peptide from HNRNPA1 validates the placement of phospho(ribose) on K350.

2.9 Supplementary Text

Note on peptide FDRs and score cutoffs:

Due to the relatively small data sets generated from peptide enrichment a standard FDR (False Discovery Rate) of 1% produces an unacceptably high FNR (False Negative Rate)[50]. Therefore alternative quality assurance conditions were applied in a parallel MaxQuant analysis where FDRs were not considered (set at 1) where scores of 40 and delta scores of 17 were set as a minimum for all peptides (MaxQuant 1.4.0.8 default settings use these cutoffs for modified peptides) and all spectra used for site identification were manually validated (see Supplementary Spectra). Peptide lists from this analysis were used for comparison of phosphoenrichment approaches (see Table 2-1, Figure 2-3 and Supplementary Figure 2-2).

Raw Files:

All raw files are hosted online: www.quantbiology.org/datasets

Spectra used in figures:

Spectra used in Figures 2-4 and 2-5 and Supplementary Figures 2-3 and 2-4 were all peptides originating from the spiked in “light” PARP-1 standard. Details:

Figure 2-4 (CID fragmentation of doubly-modified AEPVEVVAPR):

Raw file	Scan number	Modified sequence	Charge	m/z	Fragmentation	Mass analyzer	Score	Delta score
R20130702_cd_2B	7659	_AEPVEVVAPR_	2	533.79821	CID	ITMS	95.483	52.175
R20130702_cd_2B	8092	_AE(pR)PVEVVAPR_	2	639.8025	CID	ITMS	101.38	67.792
R20130702_cd_2B	8963	_AEPVE(pR)VVAPR_	2	639.8025	CID	ITMS	113.44	74.691
R20130702_cd_2B	8941	_AE(pR)PVE(pR)VVAPR_	2	745.8068	CID	ITMS	107.79	43.972

Figure 2-5 (C-terminal lysine modification):

Raw file	Scan number	Modified sequence	Charge	m/z	Fragmentation	Mass analyzer	Score	Delta score
R20130702_cd_7A	21901	_SLQELFLAHILSPWGAEVK_	2	1069.5859	CID	ITMS	212.74	185.23
R20130702_cd_2B	19264	_SLQELFLAHILSPWGAEVK(pR)_	2	1175.5902	CID	ITMS	194.15	117.39

Supplementary Figure 2-3 (HCD fragmentation of doubly-modified AEPVEVVAPR):

Raw file	Scan number	Modified sequence	Charge	m/z	Fragmentation	Mass analyzer	Score	Delta score
R20130702_cd_2B	7658	_AEPVEVVAPR_	2	533.79821	HCD	FTMS	87.895	69.223
R20130702_cd_2B	8091	_AE(pR)PVEVVAPR_	2	639.8025	HCD	FTMS	86.882	70.211
R20130702_cd_2B	8962	_AEPVE(pR)VVAPR_	2	639.8025	HCD	FTMS	86.898	74.979
R20130702_cd_2B	8940	_AE(pR)PVE(pR)VVAPR_	2	745.8068	HCD	FTMS	88.495	60.287

Supplementary Figure 2-4 (Neutral loss sequence from AEPVEVVAPR):

Raw file	Scan number	Modified sequence	Charge	m/z	Fragmentation	Mass analyzer	Score	Delta score
R20130702_cd_2B	8941	_AE(pR)PVE(pR)VVAPR_	2	745.8068	CID	ITMS	107.79	43.972

Explanation of Supplementary Tables:

Supplementary Table 2-1: phospho- and phospho(ribose) peptides identified from Casein and PARP1 standards, respectively.

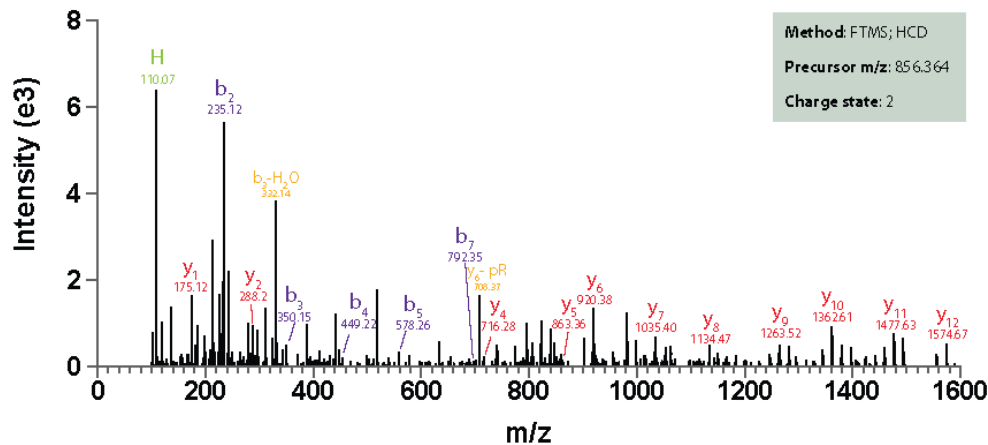
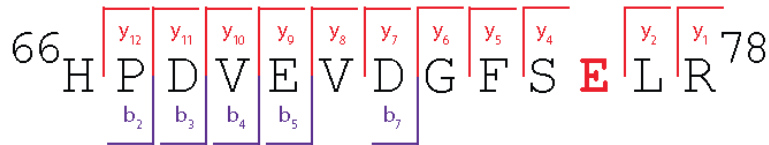
Supplementary Table 2-2: phosphopeptides identified from HeLa whole cell lysate

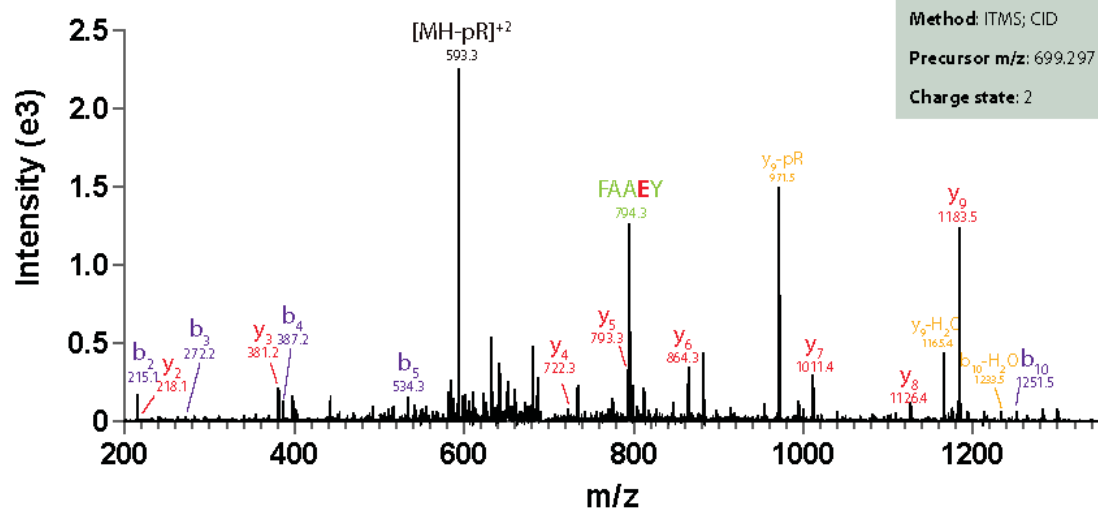
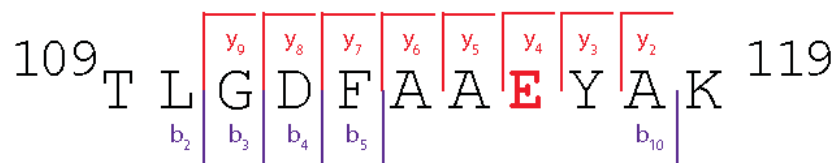
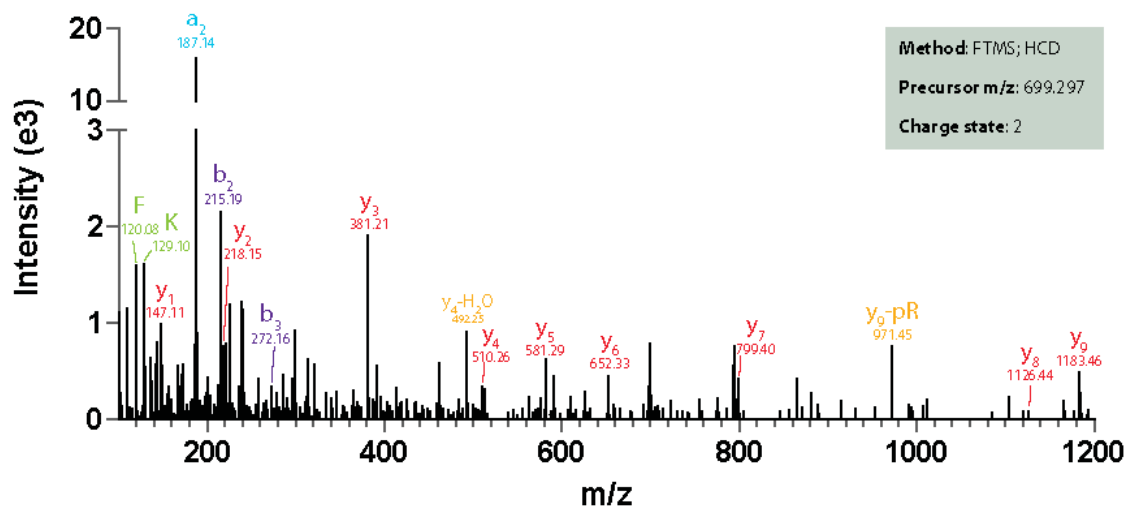
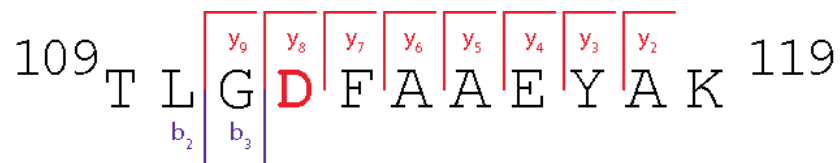
Supplementary Table 2-3: all proteins identified in this study (excluding reverse & contaminant hits)

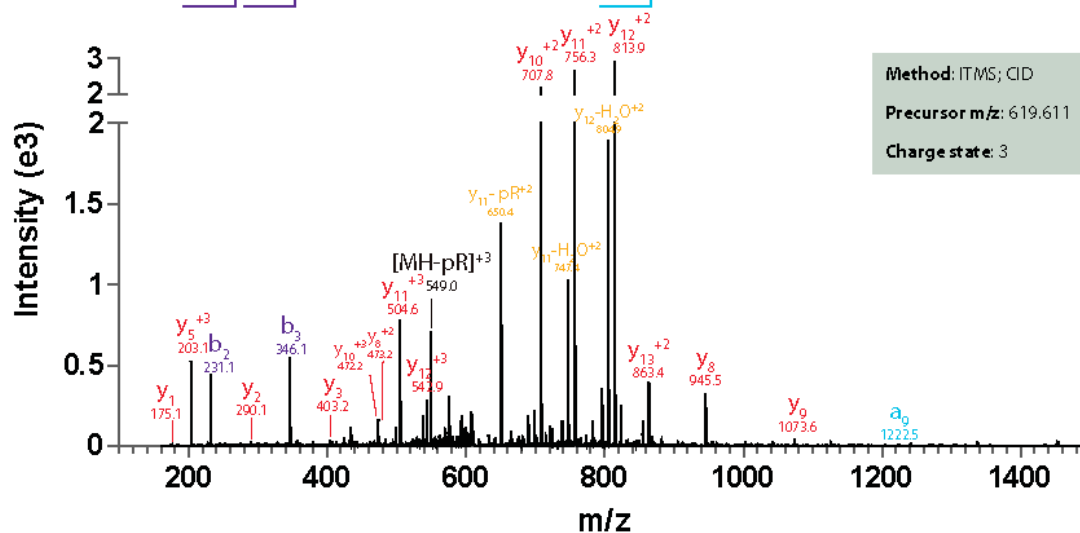
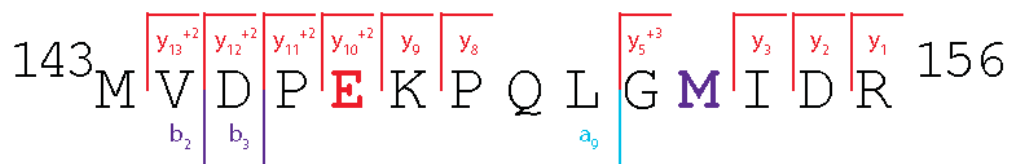
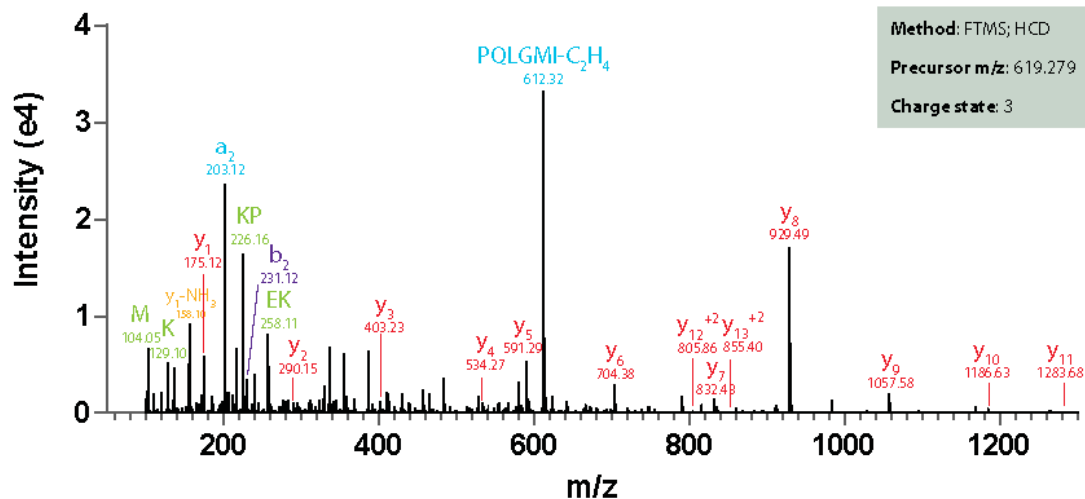
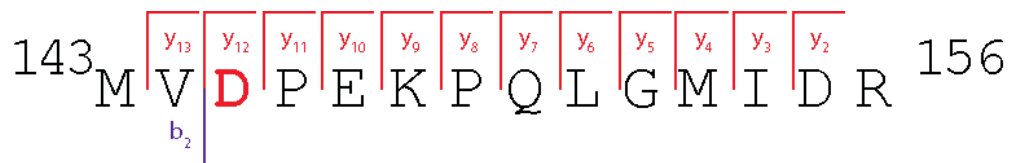
Supplementary Table 2-4: all peptides identified in this study (excluding reverse & contaminant hits)

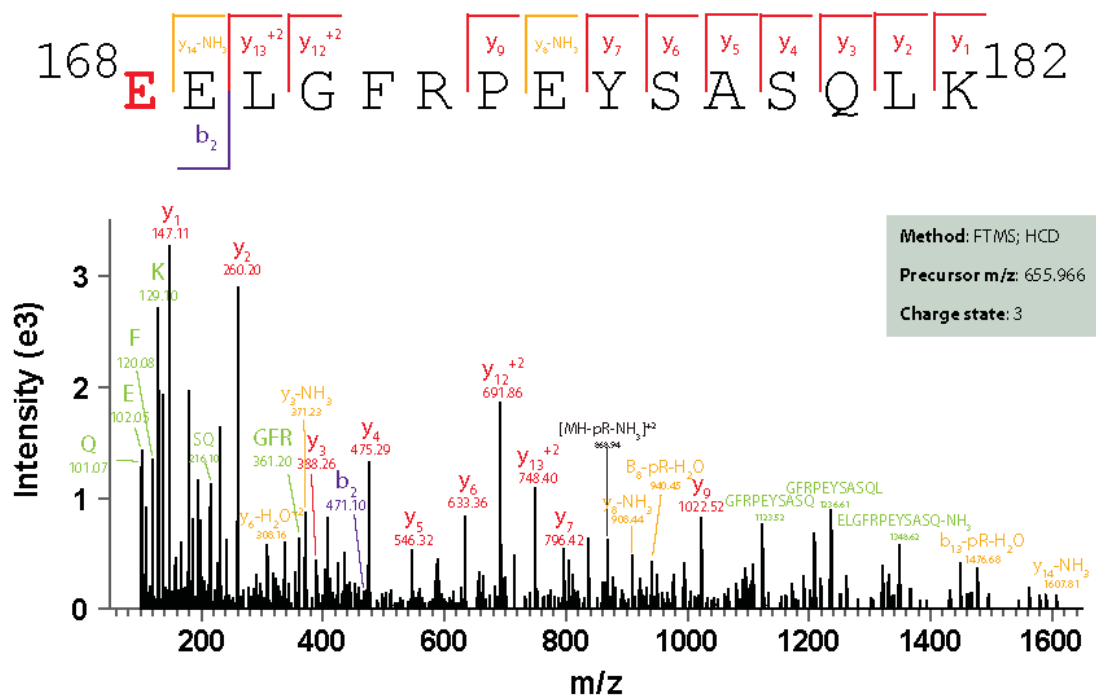
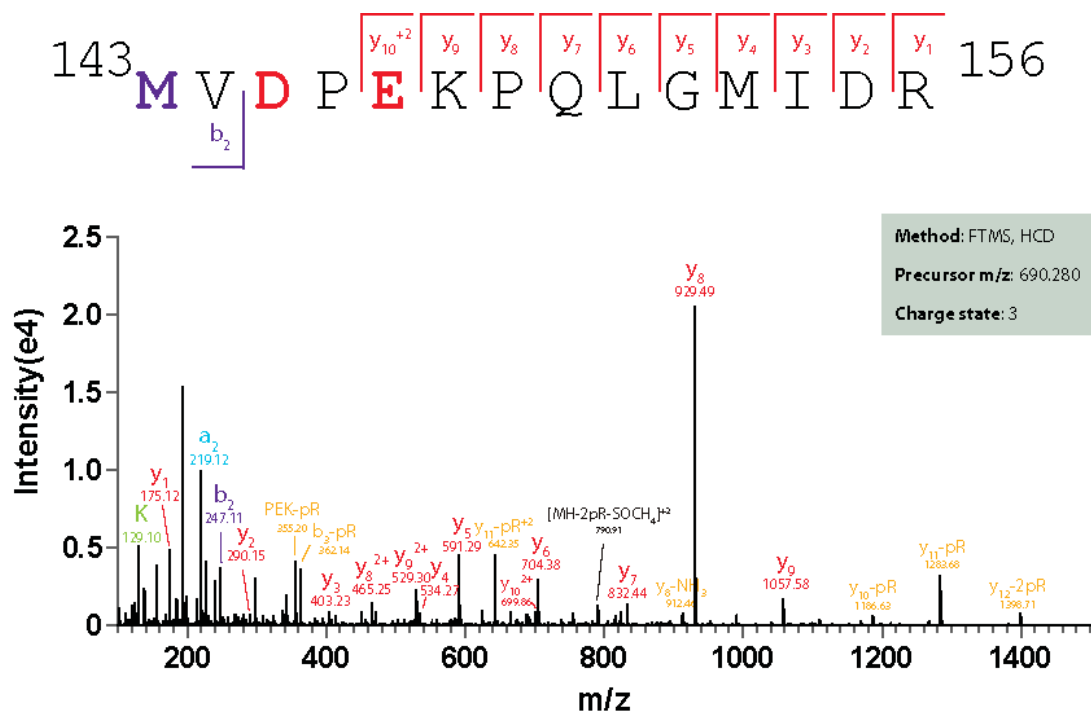
2.10 Supplementary Spectra

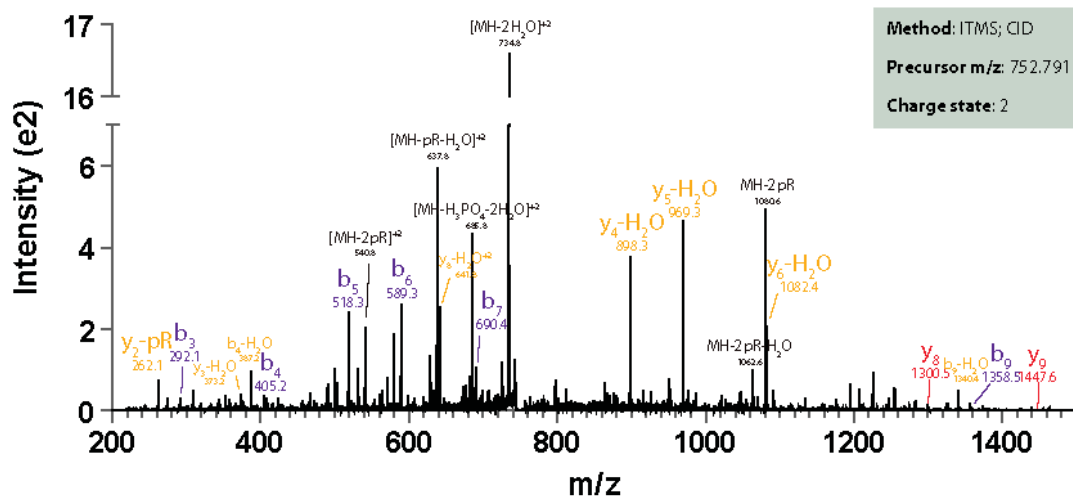
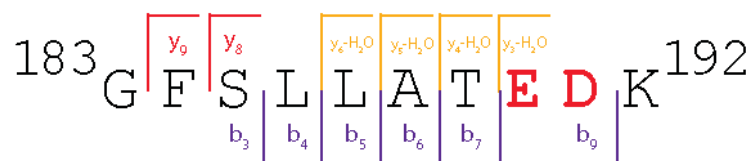
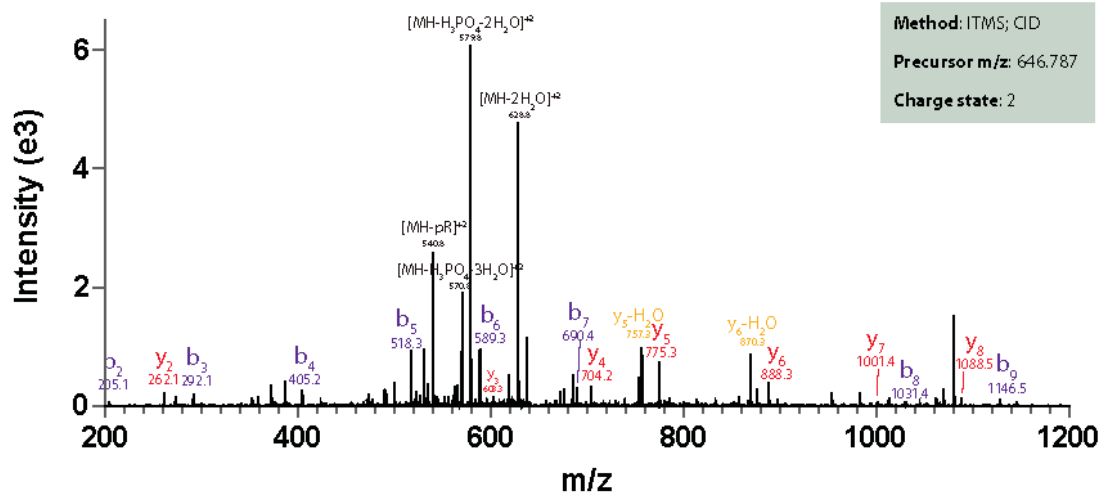
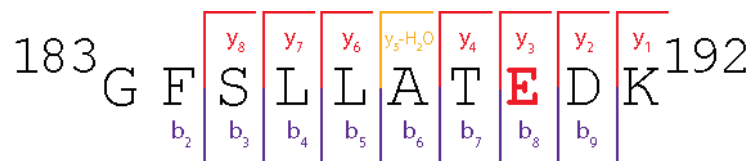
hPARP1 Automodification Sites								
Residue	precursor m/z	charge state (z)	Peptide	Raw File	Scan #	Score	Delta Score	Fragmentation
E 76	856.364	2	HPDVEVDGFSE(pR)LR	2A	10089	117.270	92.304	HCD
D 112	699.297	2	TLGD(pR)FAAEYAK	2A	10732	57.175	38.550	HCD
E 116	699.297	2	TLGDFAAE(pR)YAK	2B	11643	86.463	21.420	CID
D 145	619.279	3	MVD(pR)PEKPQLGMIDR	2B	10423	79.639	77.164	HCD
E 147	619.611	3	MVDPE(pR)KPKQLGM(ox)IDR	2A	8434	63.160	48.039	CID
E 168	655.966	3	E(pR)ELGFRPEYSASQLK	2A	9847	53.395	50.582	HCD
E 190	646.787	2	GPSLLATE(pR)DK	2A	10799	175.930	119.500	CID
D 191	752.791	2	GPSLLATE(pR)D(pR)K	7A	16174	93.598	48.575	CID
K 239	580.297	3	ALK(pR)AQNDLIWNIK	G2	12556	127.52	62.341	CID
R 452	807.379	3	EANIR(pR)VVSDFLQDVSAATK	F1	14683	244.270	170.130	CID
E 471	1175.590	2	SLQE(pR)LFLAHLSPWGAEVK	2B	19648	111.570	109.000	HCD
E 484	784.063	3	SLQELFLAHLSPWGAE(pR)VK	7B	22313	106.190	51.430	CID
K 486	1175.590	2	SLQELFLAHLSPWGAEVK(pR)	2B	19264	194.150	117.390	CID
E 488	639.803	2	AE(pR)PVEVVAPR	2B	8092	101.380	67.792	CID
E 491	639.803	2	AEPVE(pR)VVAPR	2B	8963	113.440	74.691	CID
K 498	732.361	2	AEPVEVVAPRGK(pR)	2A	6645	80.737	24.105	CID
E 619	592.261	3	LYE(pR)EKTGNAAWHSK	2B	5827	45.207	42.239	HCD
E 642	1064.456	2	FYPLE(pR)IDYGGQDEEAVK	5A	14977	95.195	89.232	HCD
D 648	1064.456	2	FYPLEIDYGQD(pR)EEAVK	2B	14375	172.090	141.850	CID
E 649	1064.456	2	FYPLEIDYGQDE(pR)EAVK	2A	13289	182.700	83.685	CID
Doubly Modified Peptides								
D 145	690.280	3	M(ox)VD(pR)PE(pR)KPKQLGMIDR	5A	10470	63.185	58.568	HCD
E 147								
E 190	752.791	2	GPSLLATE(pR)D(pR)K	7A	16174	93.598	48.575	CID
D 191								
E 488	745.807	2	AE(pR)PVE(pR)VVAPR	2B	8941	107.790	43.972	CID
E 491								

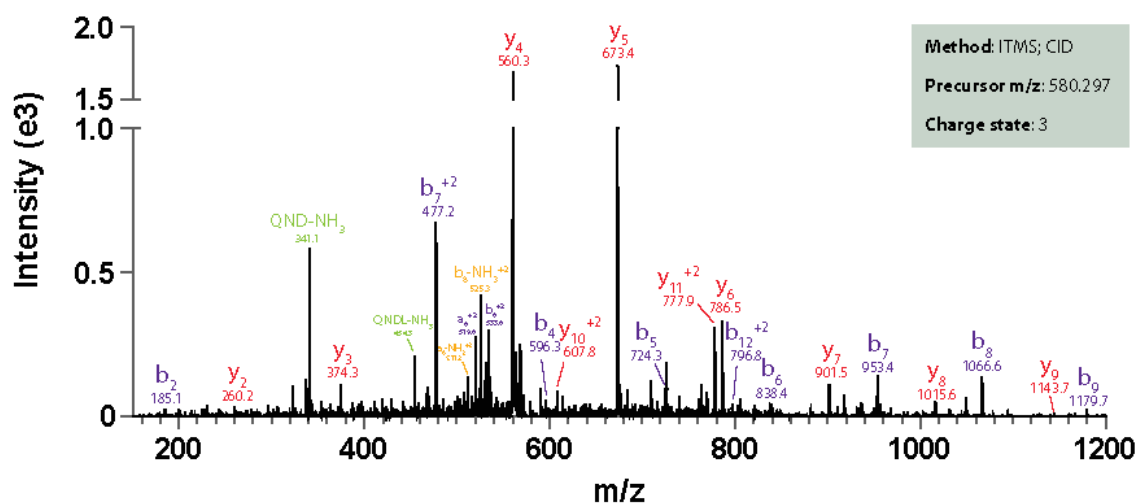
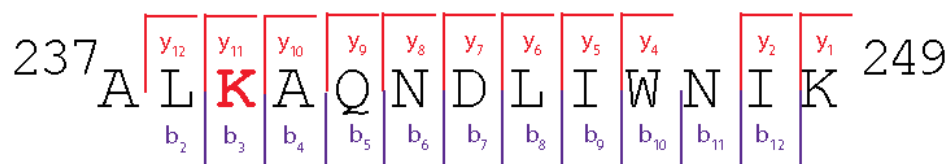
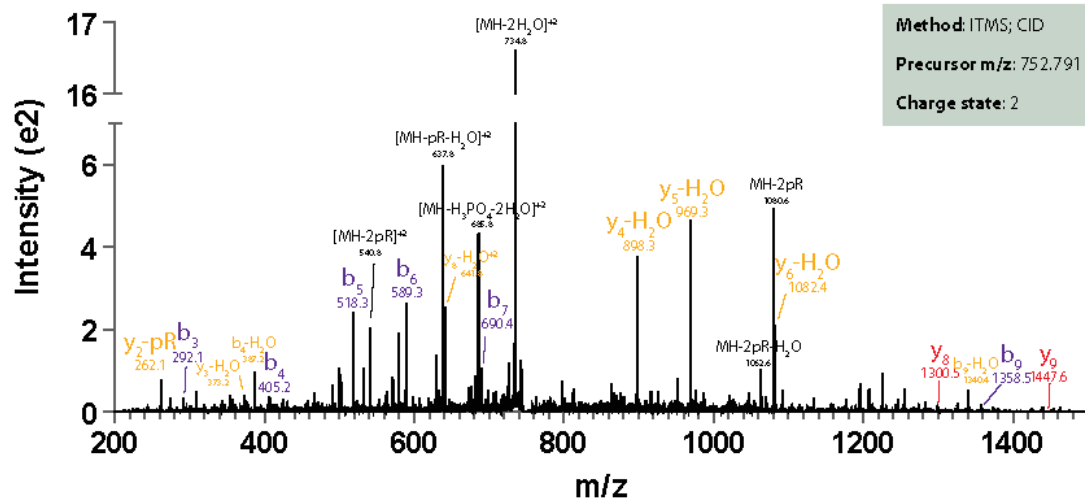
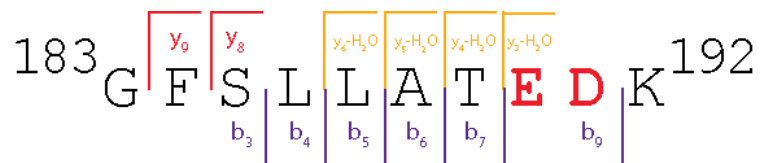


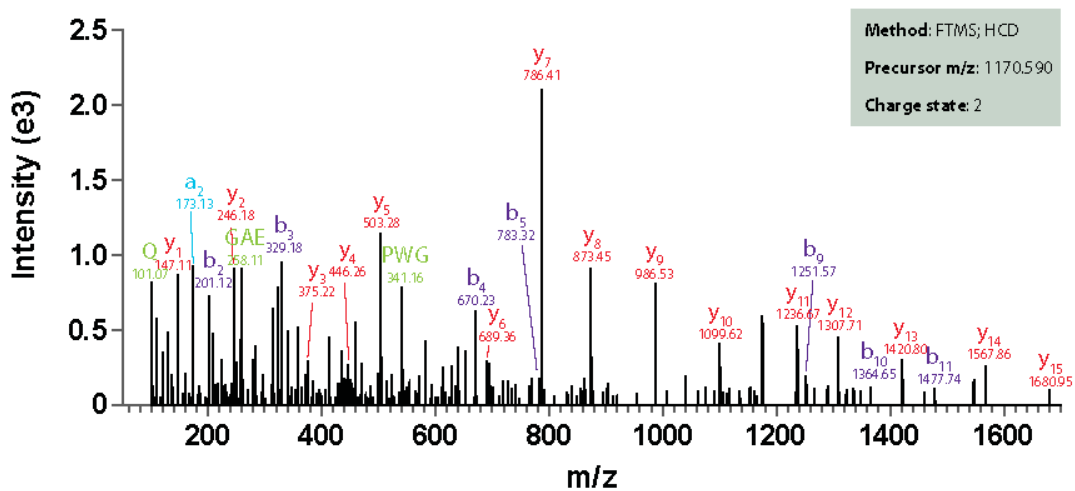
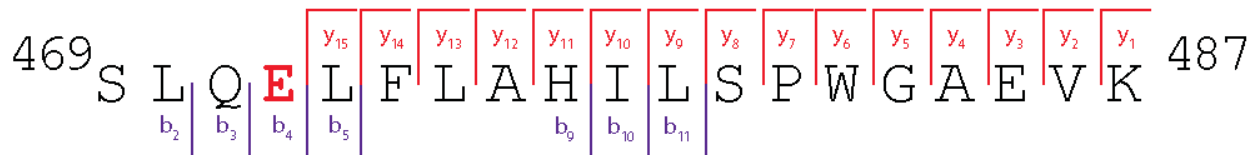
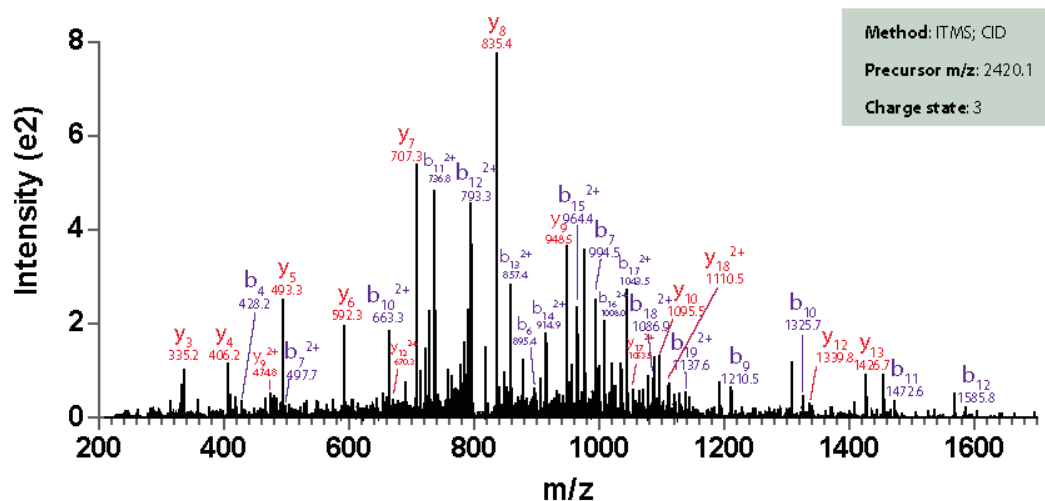
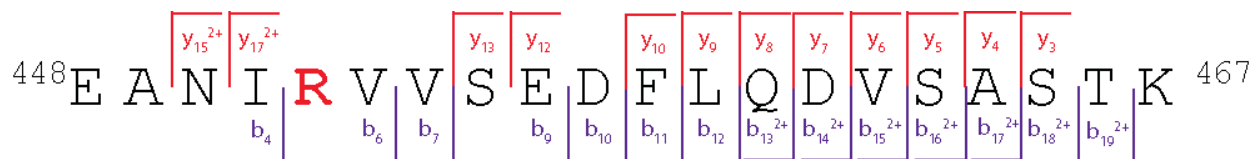


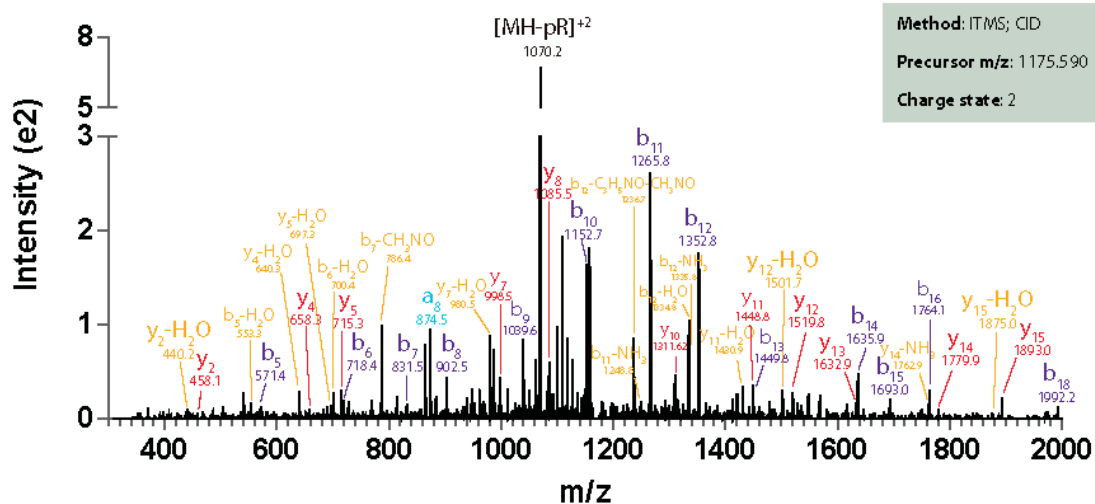
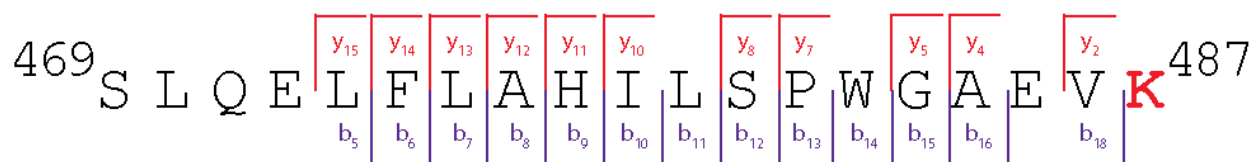
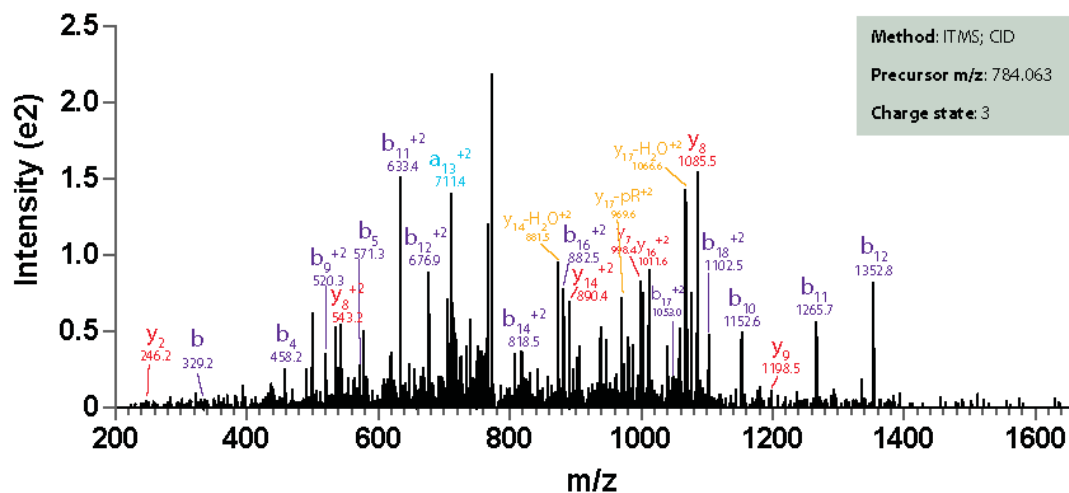
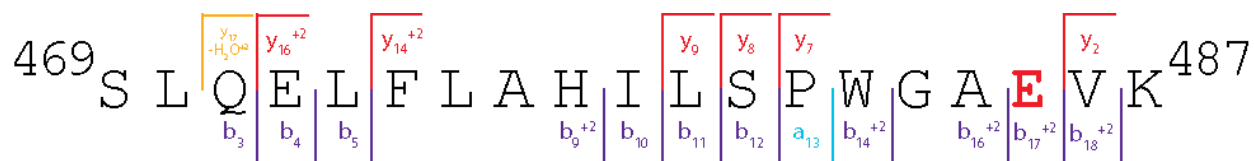


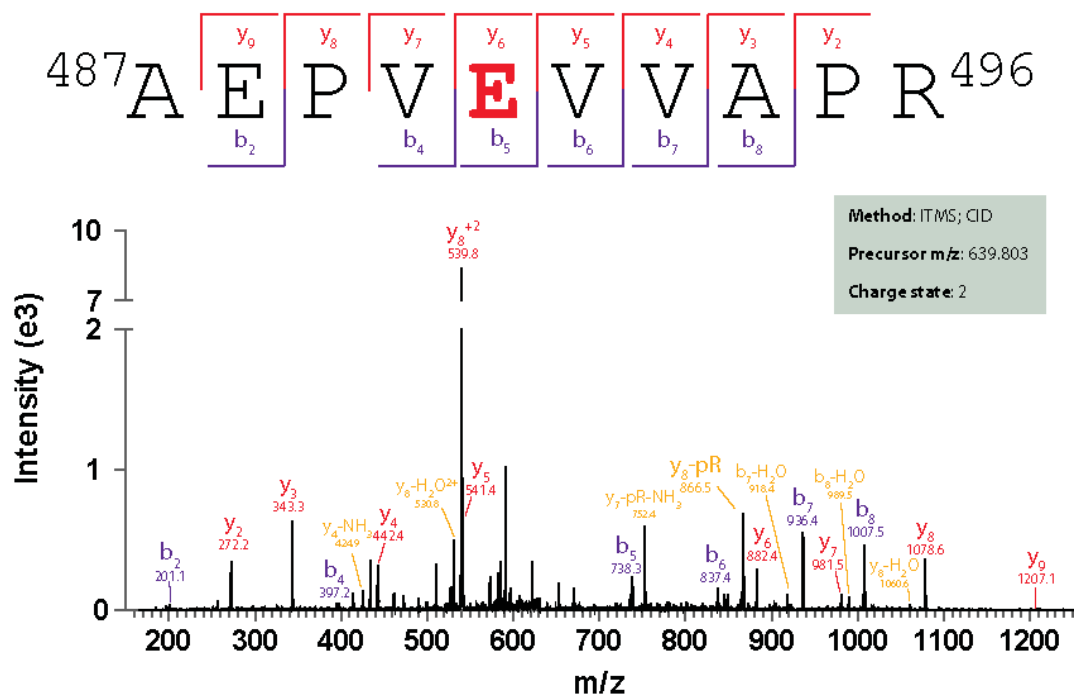
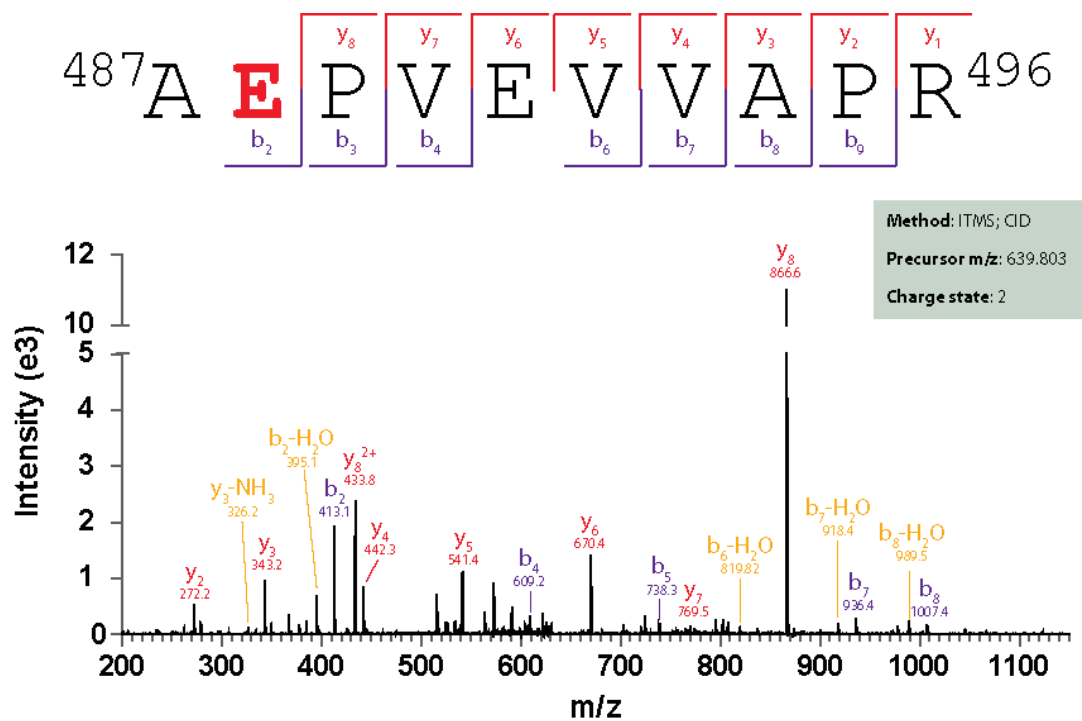


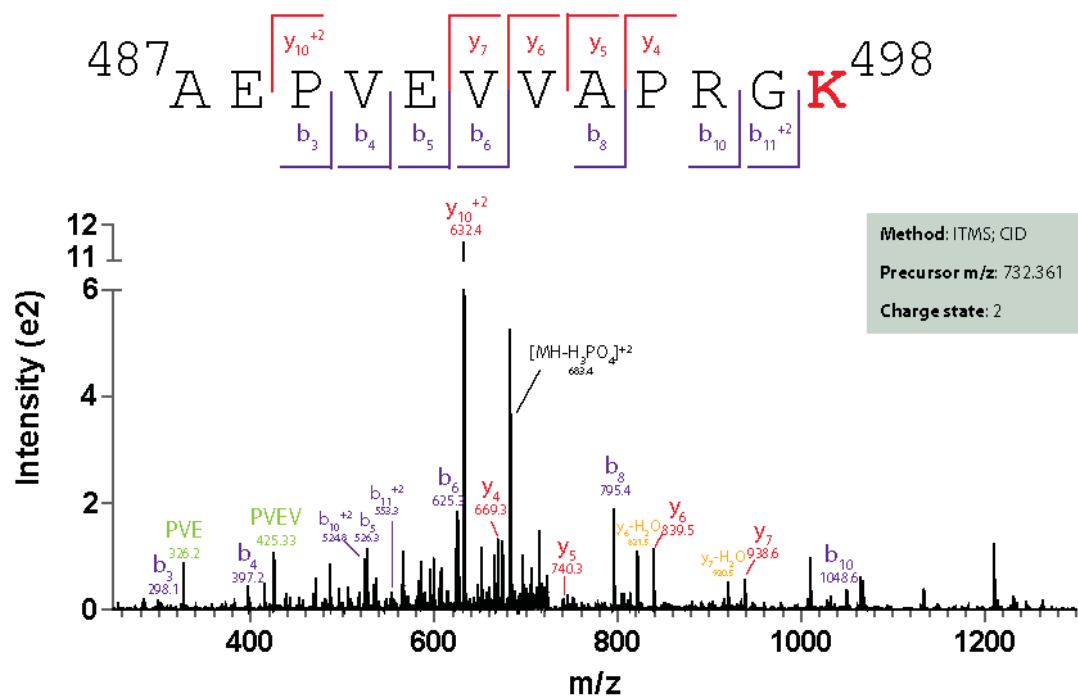
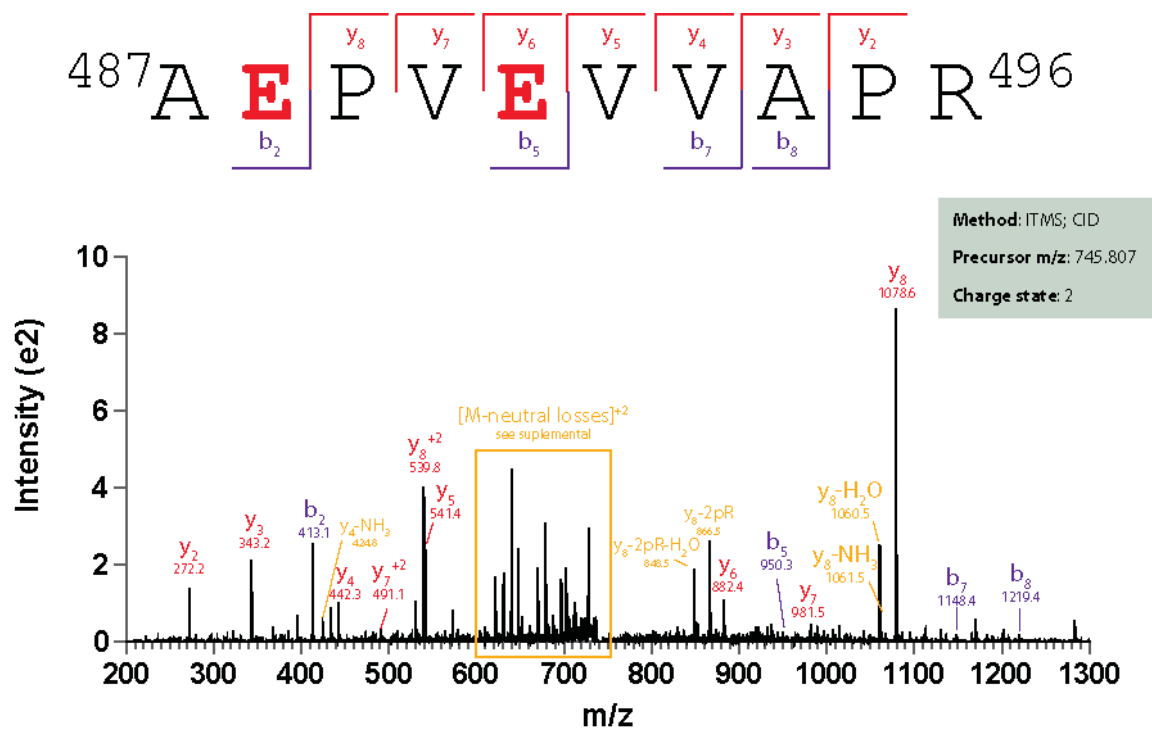


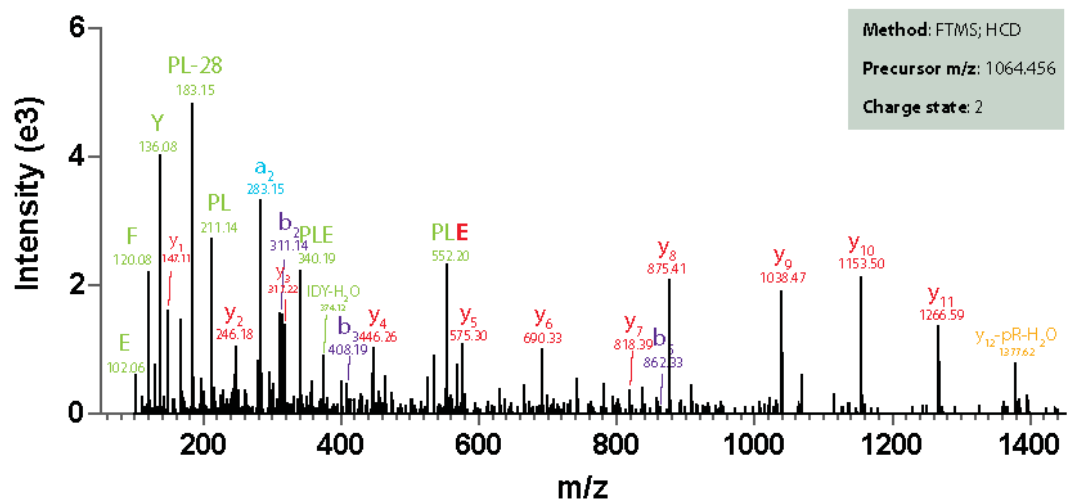
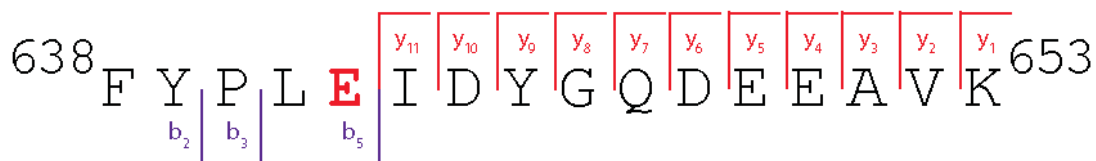
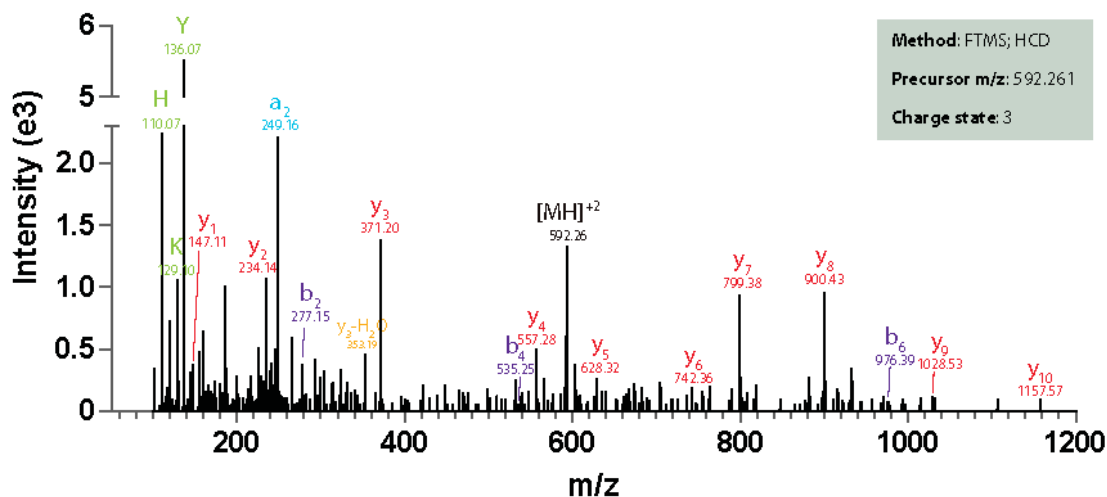
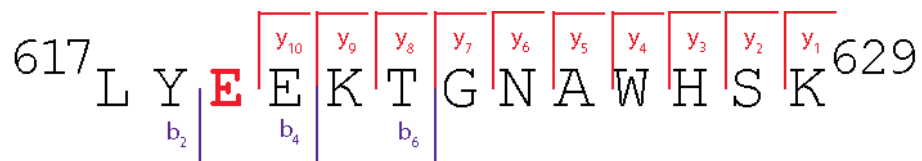


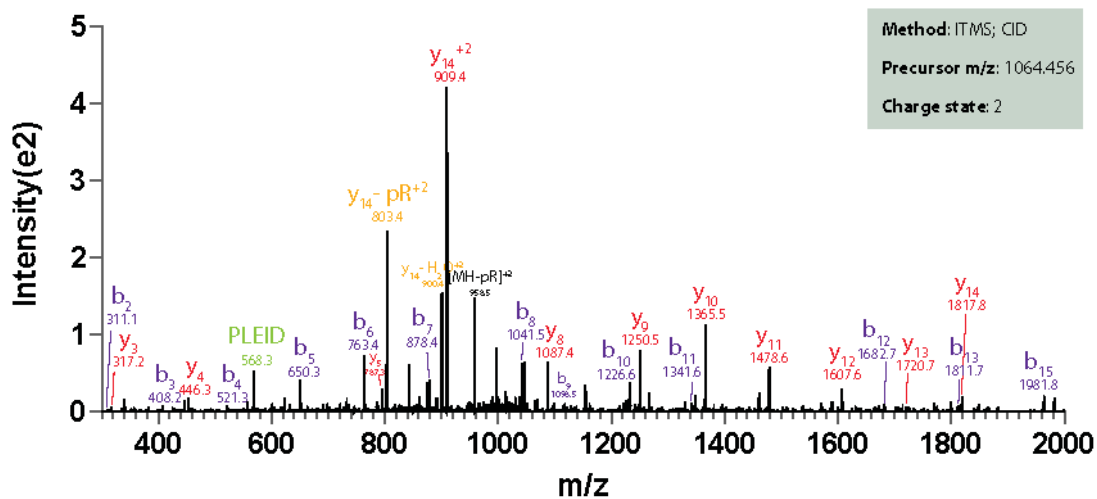
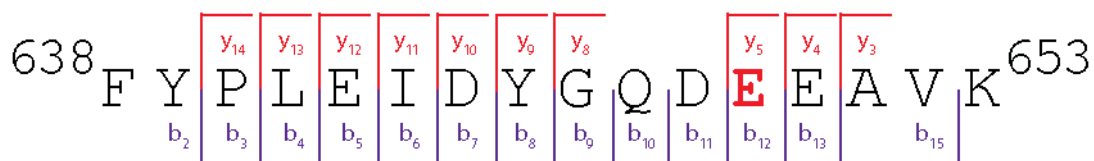
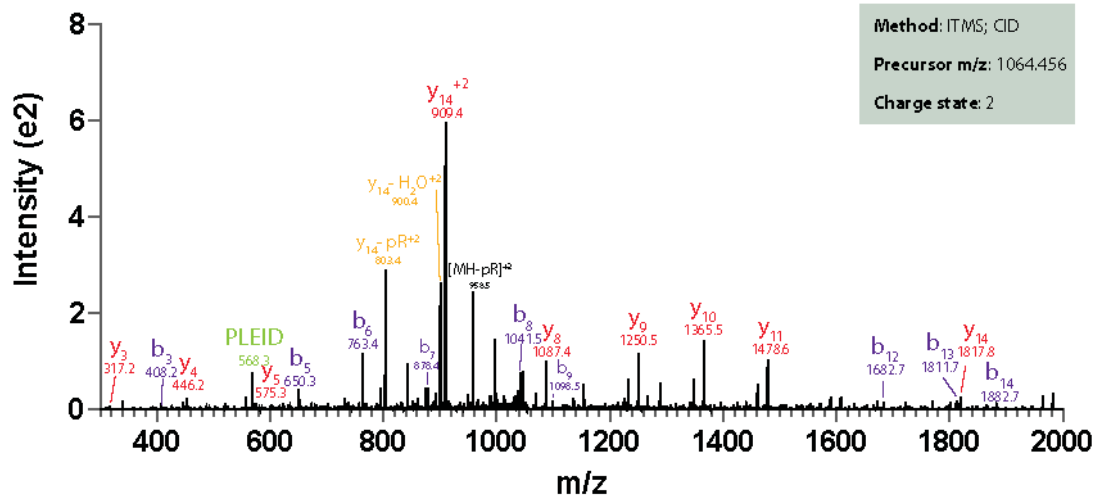
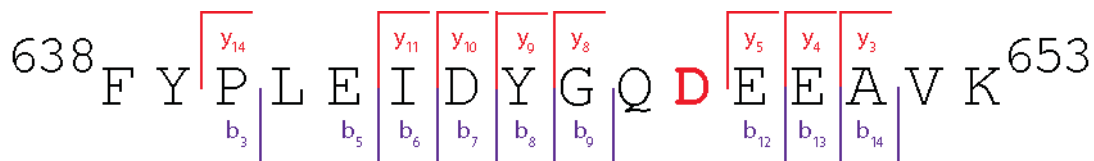












2.11 References

1. Hottiger, M.O., et al., *Toward a unified nomenclature for mammalian ADP-ribosyltransferases*. Trends Biochem Sci, 2010. **35**(4): p. 208-19.
2. De Lorenzo, S.B., et al., *The Elephant and the Blind Men: Making Sense of PARP Inhibitors in Homologous Recombination Deficient Tumor Cells*. Front Oncol, 2013. **3**: p. 228.
3. Underhill, C., M. Toulmonde, and H. Bonnefoi, *A review of PARP inhibitors: from bench to bedside*. Ann Oncol, 2011. **22**(2): p. 268-79.
4. David, K.K., et al., *Parthanatos, a messenger of death*. Front Biosci (Landmark Ed), 2009. **14**: p. 1116-28.
5. de Murcia, G., A. Huletsky, and G.G. Poirier, *Modulation of chromatin structure by poly(ADP-ribosyl)ation*. Biochem Cell Biol, 1988. **66**(6): p. 626-35.
6. Schreiber, V., et al., *Poly(ADP-ribose): novel functions for an old molecule*. Nat Rev Mol Cell Biol, 2006. **7**(7): p. 517-28.
7. Cesarone, C.F., et al., *Relationship between poly(ADP-ribose) polymerase activity and DNA synthesis in cultured hepatocytes*. Biochem Biophys Res Commun, 1990. **171**(3): p. 1037-43.
8. Smith, S., et al., *Tankyrase, a poly(ADP-ribose) polymerase at human telomeres*. Science, 1998. **282**(5393): p. 1484-7.
9. Arnold, J. and T. Grune, *PARP-mediated proteasome activation: a co-ordination of DNA repair and protein degradation?* Bioessays, 2002. **24**(11): p. 1060-5.
10. Leung, A.K., et al., *Poly(ADP-ribose) regulates stress responses and microRNA activity in the cytoplasm*. Mol Cell, 2011. **42**(4): p. 489-99.
11. Jagtap, P. and C. Szabo, *Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors*. Nat Rev Drug Discov, 2005. **4**(5): p. 421-40.
12. Feijs, K.L., P. Verheugd, and B. Luscher, *Expanding functions of intracellular resident mono-ADP-ribosylation in cell physiology*. FEBS J, 2013. **280**(15): p. 3519-29.
13. Cervantes-Laurean, D., E.L. Jacobson, and M.K. Jacobson, *Preparation of low molecular weight model conjugates for ADP-ribose linkages to protein*. Methods Enzymol, 1997. **280**: p. 275-87.
14. Zhang, Y., et al., *Site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome*. Nat Methods, 2013. **10**(10): p. 981-4.
15. Adamietz, P. and H. Hilz, *Poly(adenosine diphosphate ribose) is covalently linked to nuclear proteins by two types of bonds*. Hoppe Seylers Z Physiol Chem, 1976. **357**(4): p. 527-34.
16. Altmeyer, M., et al., *Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites*. Nucleic Acids Res, 2009. **37**(11): p. 3723-38.

17. Mao, Z., et al., *SIRT6 promotes DNA repair under stress by activating PARP1*. Science, 2011. **332**(6036): p. 1443-6.
18. Messner, S., et al., *PARP1 ADP-ribosylates lysine residues of the core histone tails*. Nucleic Acids Res, 2010. **38**(19): p. 6350-62.
19. Matsubara, H., et al., *Studies on poly (adenosine diphosphate ribose). V. Mechanism of hydrolysis of poly (adenosine diphosphate ribose) by snake venom phosphodiesterase*. J Biol Chem, 1970. **245**(14): p. 3606-11.
20. Hengel, S.M. and D.R. Goodlett, *A Review of Tandem Mass Spectrometry Characterization of Adenosine Diphosphate-Ribosylated Peptides*. Int J Mass Spectrom, 2012. **312**: p. 114-121.
21. Matic, I., I. Ahel, and R.T. Hay, *Reanalysis of phosphoproteomics data uncovers ADP-ribosylation sites*. Nat Methods, 2012. **9**(8): p. 771-2.
22. Huttlin, E.L., et al., *A tissue-specific atlas of mouse protein phosphorylation and expression*. Cell, 2010. **143**(7): p. 1174-89.
23. Chapman, J.D., et al., *Mapping PARP-1 auto-ADP-ribosylation sites by liquid chromatography-tandem mass spectrometry*. J Proteome Res, 2013.
24. Langelier, M.F., et al., *Purification of human PARP-1 and PARP-1 domains from Escherichia coli for structural and biochemical analysis*. Methods Mol Biol, 2011. **780**: p. 209-26.
25. Oka, J., K. Ueda, and O. Hayaishi, *Snake venom phosphodiesterase: simple purification with Blue Sepharose and its application to poly(ADP-ribose) study*. Biochem Biophys Res Commun, 1978. **80**(4): p. 841-8.
26. Koh, D.W., et al., *Failure to degrade poly(ADP-ribose) causes increased sensitivity to cytotoxicity and early embryonic lethality*. Proc Natl Acad Sci U S A, 2004. **101**(51): p. 17699-704.
27. Rappsilber, J., Y. Ishihama, and M. Mann, *Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics*. Anal Chem, 2003. **75**(3): p. 663-70.
28. Villen, J. and S.P. Gygi, *The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry*. Nat Protoc, 2008. **3**(10): p. 1630-8.
29. Ong, S.E., et al., *Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics*. Mol Cell Proteomics, 2002. **1**(5): p. 376-86.
30. Desmarais, Y., et al., *Enzymological properties of poly(ADP-ribose)polymerase: characterization of automodification sites and NADase activity*. Biochim Biophys Acta, 1991. **1078**(2): p. 179-86.
31. Langelier, M.F., et al., *Crystal structures of poly(ADP-ribose) polymerase-1 (PARP-1) zinc fingers bound to DNA: structural and functional insights*

- into DNA-dependent PARP-1 activity. *J Biol Chem*, 2011. **286**(12): p. 10690-701.
32. Menard, L., L. Thibault, and G.G. Poirier, *Reconstitution of an in vitro poly(ADP-ribose) turnover system*. *Biochim Biophys Acta*, 1990. **1049**(1): p. 45-58.
 33. Kharadia, S.V. and D.J. Graves, *Relationship of phosphorylation and ADP-ribosylation using a synthetic peptide as a model substrate*. *J Biol Chem*, 1987. **262**(36): p. 17379-83.
 34. Karras, G.I., et al., *The macro domain is an ADP-ribose binding module*. *EMBO J*, 2005. **24**(11): p. 1911-20.
 35. Rosenthal, F., et al., *Macrodomain-containing proteins are new mono-ADP-ribosylhydrolases*. *Nat Struct Mol Biol*, 2013. **20**(4): p. 502-7.
 36. Jankevicius, G., et al., *A family of macrodomain proteins reverses cellular mono-ADP-ribosylation*. *Nat Struct Mol Biol*, 2013. **20**(4): p. 508-14.
 37. Vyas, S., et al., *A systematic analysis of the PARP protein family identifies new functions critical for cell physiology*. *Nat Commun*, 2013. **4**: p. 2240.
 38. Gagne, J.P., et al., *Quantitative proteomics profiling of the poly(ADP-ribose)-related response to genotoxic stress*. *Nucleic Acids Res*, 2012. **40**(16): p. 7788-805.
 39. Jungmichel, S., et al., *Proteome-wide Identification of Poly(ADP-Ribosylation) Targets in Different Genotoxic Stress Responses*. *Mol Cell*, 2013.
 40. Aryal, U.K. and A.R. Ross, *Enrichment and analysis of phosphopeptides under different experimental conditions using titanium dioxide affinity chromatography and mass spectrometry*. *Rapid Commun Mass Spectrom*, 2010. **24**(2): p. 219-31.
 41. Park, S.S. and S. Maudsley, *Discontinuous pH gradient-mediated separation of TiO₂-enriched phosphopeptides*. *Anal Biochem*, 2011. **409**(1): p. 81-8.
 42. Hart, S.R., et al., *Factors governing the solubilization of phosphopeptides retained on ferric NTA IMAC beads and their analysis by MALDI TOFMS*. *J Am Soc Mass Spectrom*, 2002. **13**(9): p. 1042-51.
 43. Schulenberg, B., et al., *Characterization of dynamic and steady-state protein phosphorylation using a fluorescent phosphoprotein gel stain and mass spectrometry*. *Electrophoresis*, 2004. **25**(15): p. 2526-32.
 44. Kostka, G. and A. Schweiger, *ADP-ribosylation of proteins associated with heterogeneous nuclear RNA in rat liver nuclei*. *Biochim Biophys Acta*, 1982. **696**(2): p. 139-44.
 45. Prasad, S., J. Walent, and A. Dritschilo, *ADP-ribosylation of heterogeneous ribonucleoproteins in HeLa cells*. *Biochem Biophys Res Commun*, 1994. **204**(2): p. 772-9.

46. Ji, Y. and A.V. Tulin, *Poly(ADP-ribosyl)ation of heterogeneous nuclear ribonucleoproteins modulates splicing*. Nucleic Acids Res, 2009. **37**(11): p. 3501-13.
47. Ji, Y. and A.V. Tulin, *Poly(ADP-ribose) controls DE-cadherin-dependent stem cell maintenance and oocyte localization*. Nat Commun, 2012. **3**: p. 760.
48. Tao, Z., P. Gao, and H.W. Liu, *Identification of the ADP-ribosylation sites in the PARP-1 automodification domain: analysis and implications*. J Am Chem Soc, 2009. **131**(40): p. 14258-60.
49. Sharifi, R., et al., *Deficiency of terminal ADP-ribose protein glycohydrolase TARG1/C6orf130 in neurodegenerative disease*. EMBO J, 2013. **32**(9): p. 1225-37.
50. Pawitan, Y., et al., *False discovery rate, sensitivity and sample size for microarray studies*. Bioinformatics, 2005. **21**(13): p. 3017-24.

3 Nudix Hydrolases degrade protein-conjugated ADP-ribose

Casey M. Daniels¹, Puchong Thirawatananond², Shao-En Ong³, Sandra B. Gabelli^{2,4,5}, and Anthony K. L. Leung¹

¹Department of Biochemistry and Molecular Biology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA.

²Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

³Department of Pharmacology, University of Washington, Seattle, WA 98195, USA.

⁴Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205, USA.

⁵Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205, USA.

3.1 Abstract

Poly(ADP-ribose) and mono(ADP-ribose) (collectively: ADP-ribose, ADPr) are post-translational modifications (PTMs) important in all major cellular processes, poly(ADP-ribose) is particularly well known for the critical role it plays in DNA repair, and consequently, cancer. Study of this PTM has been limited, however, by a lack of mass spectrometry based proteomic tools for identifying the amino acid residues carrying this modification. Recent work from our group and others has demonstrated the potential of a tag-based pipeline in which the ADPr monomer or polymer is hydrolyzed down to its phosphoribose protein attachment site, leaving a 212 Dalton tag at the site of modification. While the pipeline has been proven effective by multiple groups, a barrier to application has become evident: the enzyme used to transform ADPr into phosphoribose – snake venom phosphodiesterase (SVP) from the rattlesnake *Crotalus adamanteus* – must be purified from venom (no recombinant expression/purification scheme has been shown) and can be tedious to isolate away from all contaminating phosphatases and proteases. In this study we outline the steps necessary for successfully purifying SVP for use in this pipeline and also present two alternatives to SVP, both from the Nudix hydrolase super family: *HsNudT16* and *ErRppH*. Importantly, expression and purification schemes for these enzymes have already been proven, with large, high-quality yields easily attainable. Here we have demonstrated their utility in identifying ADP-ribosylation sites on Poly(ADP-ribose) Polymerase 1 (PARP1) by mass spectrometry and have offered structure-based rationale for the ability of these Nudix hydrolases, but not Nudix ADPrases, to degrade protein-conjugated ADPr.

3.2 Introduction

ADP-ribosylation is a post-translational modification implicated in a number of disease states, including cancer, diabetes, and a range of neuropathologies [1]. This protein modification is synthesized by ADP-ribosyl transferases, commonly known as PARPs (poly-ADPr-polymerases), which transfer the ADP-ribose (ADPr) group from NAD⁺ to protein acceptor amino acids in monomeric (mono(ADPr), MAR) and/or polymeric (poly(ADPr), PAR) form [2, 3]. Identification of

specific amino acid acceptors of ADPr, and therefore characterization of the cellular role played by this important protein modification, has been hampered by the lack of a robust, universal method for both identifying all ADP-ribosylation sites in the proteome as well as individual sites on known acceptor proteins. This need has lately been addressed by three different methods, all of which involve the digestion or removal of the polymer followed by identification of the ‘tag’ left behind at the ADPr conjugation site [4]. One of these methods, digestion of ADPr down to its phosphoribose (pR) attachment site, relies upon the pyrophosphatase activity of snake venom phosphodiesterase I (SVP) from *Crotalus adamanateus*, an enzyme which can be purchased in a partially purified form and further purified for use against ADP-ribosylated proteins [3, 5, 6]. Unfortunately, this complicated purification scheme ultimately results in a high level of prep-to-prep variability, likely due to the inherently variable protein source (snake venom) as well as the number of purification steps involved. In an effort to identify a more reliable tool for the degradation of protein-conjugated ADPr to phosphoribose, we describe here the characterization of candidate enzymes from the Nudix hydrolase superfamily.

The Nudix hydrolase superfamily catalyzes hydrolysis of **N**ucleoside **D**iphosphates linked to other moieties (“**X**”). Most Nudix families contribute to cellular ‘housekeeping’ through the breakdown of a wide range of nucleoside diphosphate derivatives [7]. One of these diphosphate containing compounds is ADPr [8, 9], a molecule which can accumulate in cells with potentially cytotoxic effects by: (1) altering calcium entry into cells via channel gating, thus affecting membrane depolarization [10], (2) serving as a neurotransmitter in primate and murine colons [11], and (3) spontaneously modifying proteins [12], potentially altering intracellular post-translational signaling. Without Nudix hydrolase activity free ADPr would amass during the breakdown of PAR [13, 14], as a side product of tRNA synthesis [15], following NAD⁺ glycohydrolysis [16], following deacetylation of O-acetyl-ADPr [17], and through the breakdown of the signaling molecule cyclic ADPr [18]. Accordingly, ADPr degrading Nudix enzymes are broadly conserved, with humans possessing at least six distinct ADPr pyrophosphatases (ADPrases) [19, 20]. In searching for a Nudix hydrolase

substitute for SVP we screened bacterial Nudix hydrolases and identified *Ec*RppH as capable of degrading protein conjugated ADPr and thus providing a phosphoribose tag identifiable by mass spectrometry. From a structural and biological perspective this finding was intriguing as *Ec*RppH is an RNA decapping enzyme, and not an ADPrase [21]. Not long after we made this discovery *Hs*NudT16, a human Nudix hydrolase also known to decap RNA [22], was shown to degrade protein conjugated MAR and PAR [23]. In this study we provide structure-based rationale for the inability of Nudix ADPrases to degrade protein conjugated PAR/MAR, in contrast to Nudix RNA decapping enzymes, and demonstrate the use of both *Ec*RppH and *Hs*NudT16 in the identification of protein ADP-ribosylation sites by mass spectrometry.

3.3 Materials and Methods

Mutagenesis of PARP1 to the E988Q catalytically deficient mutant

pET28 His-PARP1 was a gift from Dr. John Pascal and served as the template for mutagenesis into the mono(ADP-ribose) restricted mutant of PARP1, E988Q. Mutagenesis took place with the following final concentrations: 1x Pfu reaction buffer (Agilent), 0.5 ng/μL pET28 His-PARP1 template, 2.5 ng/μL primers (Forward:

GACACCTCTCTACTATATAAC**C**AGTACATTGTCTATGATATTGC, Reverse:

GCAATATCATAGACAATGTACT**G**GTTATATAGTAGAGAGGTGTC), 0.2 mM dNTPs (Life Technologies), 1 μL of *Pfu*Turbo DNA polymerase (Agilent), the PCR method was as follows: 95°C for 30 seconds (1 cycle), 95°C for 30 seconds/55°C for 60 seconds/68°C for 17 minutes (12 cycles), 68°C for 51 minutes (1 cycle). The template was digested with 1 μL (10 units) of DpnI restriction enzyme (New England Biolabs) for 90 minutes at 37°C. 5 μL of the digested DNA was transferred to one tube of SoloPack Gold Supercompetent Cells (Stratagene) and incubated on ice for 30 minutes, placed in a 42°C water bath for 30 seconds and then placed on ice for 2 minutes. 250 μL of pre-heated SOC medium (Quality Biological) was added to the reaction and incubated for 1 hr at

37⁰C shaking at 250 rpm. Cells were plated on LB-Kanamycin plates and incubated at 37⁰C overnight. Colonies were sequenced for validation.

Expression and purification of WT & E988Q HisPARP1

Method was performed as published previously [5].

Purification of SVP

Method was performed as published previously [5]. Snake venom phosphodiesterase was obtained from United States Biological, catalog number P4072, lot number L14030507 C14062702.

Assessment of contaminating proteolysis activity in SVP prep

For whole cell lysate (Figure 1e) 1 mg of proteins from HeLa whole cell lysate was denatured in 8M Urea (Sigma-Aldrich) 50 mM Tris pH 7 for 10 minutes at 37⁰C before being reduced in 1 mM Tris-(2-Carboxyethyl)phosphine (Life Technologies) for 10 minutes and then alkylated in 2 mM 2-chloroacetamide (Sigma-Aldrich) for 10 minutes in the dark. Samples were then diluted to a final concentration of 1M Urea, 50 mM NaCl (Sigma-Aldrich), 15 mM MgCl₂ (Quality Biological) and 0.2M Tris pH 7.3. 5 µg of SVP was added to each sample and incubated for 2 hours at 37⁰C. Samples were run on an in-house 6-10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Total protein was visualized by ProAct membrane stain (Amresco) per the manufacturer's instructions. For purified recombinant His-PARP1, 1 µg of His-PARP1 was automodified as previously published [5] and then switched into the same buffer used for the whole cell lysate (above) with or without 1M urea. 500 ng of SVP was used for each µg of PARP1 and digestion was carried out at 37⁰C for 2 hours. Samples were run on in-house 6-10% SDS-PAGE gels and total protein was visualized by SimplyBlue Safe Stain (Life Technologies) per the manufacturer's instructions. ³²P-labeled PAR was visualized on a phosphor-screen (GE,BAS-III 2040) followed by imaging on a Typhoon FLA7000 (GE Healthcare Life Sciences).

Expression and purification of Nudix hydrolases

The expression and purification have been previously published for the following hydrolases:

*Ec*NudF/ADPRase [20], *Ec*NudE [24], *Bd*3179/*Bd*NDPSase [25], *Dr*1184 [26], *Ec*RppH [27], *At*ORF147 and *Pa*3470 [28]. *Hs*NudT16 was expressed and purified as described in the methods from the Structural Genomics Consortium [29]. *Ec*YfcD was purified using the method described for *Ec*ADPRase [20].

Structural analysis of Selected Nudix hydrolases

Selected Nucleotide sugar hydrolases were used as stereotypes of families according to their published preferred substrate ADPr for *Ec*ADPRase, *Tb*ADPRase, *Hs*ADPRase; GDP-mannose for *Ec*GDPMK; and *Bd*NDPSase as a general sugar hydrolase. The structures were structurally aligned using SSM and rendered using PyMOL [30]. PAR was constructed and minimized in MOE. For the sugar hydrolases PAR was modeled in the active site taking into account the binding preference observed in the structures.

Selected known monomeric Nudix enzymes were structurally aligned using SSM and rendered using PyMOL. PAR was modeled in the active site using the mRNA present in NudT16.

Automodification of WT and E988Q PARP1

Automodification was performed as published previously [5] with the following changes: both wildtype and E988Q PARP1 were incubated with 0.6 μ M (0.05 μ Ci/ μ L, 1 μ Ci/sample) 32 P-NAD for 10 minutes at room temperature, following which wildtype PARP1 was incubated with 1 mM NAD (non-radioactive) for 10 minutes at room temperature to allow for polymer elongation.

Digestion of ADPr to phosphoribose

5 pmoles of PARP1 wildtype or E988Q mutant were exposed to a hydrolase (various enzymes and amounts) in hydrolysis buffer (50 mM Tris pH 7 (Thermo Scientific), 150 mM NaCl (Sigma-Aldrich), 15 mM MgCl₂ (Quality Biological), 1 mM 3-aminobenzamide (Sigma Aldrich) for two hours at 37⁰C.

Protein digestions for LC-MS/MS analysis

Proteins were denatured in 8 M Urea (Sigma Aldrich) 50 mM Tris pH 7 for 10 minutes at 37⁰C before being reduced in 1 mM Tris-(2-Carboxyethyl)phosphine (Life Technologies) for 10 minutes and then alkylated in 2 mM 2-chloroacetamide (Sigma-Aldrich) for 10 minutes in the dark. Samples were diluted to: 1M Urea, 50 mM NaCl (Sigma-Aldrich), 15 mM MgCl₂ (Quality Biological), 0.2 M Tris pH 7 (7.3 at room temperature), and 1 mM CaCl₂ (Sigma-Aldrich). LysC (Wako) and Trypsin (Promega) were added at in a 1:50 enzyme:substrate ratio.

Phosphoenrichment of phosphoribosylated peptides

The ion metal affinity chromatography (IMAC) method described by us in [5] was used.

LC-MS/MS analysis of phosphoribosylated peptides

Analysis was performed as in [5].

Database search of MS/MS spectra for peptide and protein identification

Raw files were analyzed by MaxQuant version 1.5.3.8 using protein, peptide and site FDRs of 0.01 and a score minimum of 40 for modified peptides, 0 for unmodified peptides; delta score minimum of 17 for modified peptides, 0 for unmodified peptides. Sequences were searched against an in-house database containing the proteins of interest as well as Uniprot *Escherichia coli* BL21 DE3 database (definitions updated October 15th, 2014). MaxQuant search parameters: Variable modifications included Oxidation (M), Acetylation (Protein N-term), carbamidomethyl (C), phosphorylation (STY) and phosphoribosylation (DEKRC). Max labeled amino acids was 3, max missed cleavages was 2, enzyme was Trypsin/P, and max charge was 7.

3.4 Results

Purification of Phosphodiesterase I from *Crotalus adamanteus* involves both affinity purification and size exclusion chromatography

Snake venom phosphodiesterase I (SVP) from *Crotalus adamanteus* was shown to degrade PAR nearly 50 years ago [31] and has since proven a valuable tool for the degradation of PAR into its linear, branching and terminal subunits, a technique that yields quantitative information regarding the molecular structure of the intact polymer [32, 33]. The utility of this enzyme, however, is greatly determined by the purification scheme employed to isolate it from the large number of proteases as well as phosphatases and nucleotidases present in the *C. adamanteus* venom [34]. Oka *et al* successfully isolated the phosphodiesterase activity of commercially available SVP away from the contaminating phosphatase and 5'-nucleotidase activity through affinity purification using blue sepharose, a molecule which mimics NAD⁺ and therefore interacts with the active domain of SVP [35]. A simplified version of this method used by our group is shown in Fig. 3-1a, where 150 mM Potassium Phosphate pH 7.5 is used as a single step elution off of a blue sepharose column. This purification scheme paved the way for development of the quantitative method mentioned above but did not address the need to eliminate contaminating protease activity. This protease activity can be problematic when using SVP to digest protein-conjugated ADPr (e.g. for the purpose of creating a phosphoribose 'tag' at the otherwise ADP-ribosylated amino acid residue [5, 6, 36]), as we have shown in figure 1e wherein a complex mixture of proteins is exposed to blue sepharose purified SVP resulting in the degradation of the target proteins and the appearance of SVP along with its co-purified proteins. This proteolytic activity is further shown against purified, ³²P-PARylated PARP1 (both native and denatured) in Fig. 3-1f. In order to separate the 115 kD SVP from the major contaminating proteins (<30 kD) we subjected the blue sepharose purified product to size exclusion chromatography, yielding a simple mixture of what are presumably the various glycolytic forms of SVP (Fig. 3-1b-d). When tested against ³²P-PARylated PARP1 as in Fig. 3-1f this highly pure form of SVP displayed phosphodiesterase activity without apparent proteolytic activity (Fig. 3-1g). Similar

results were seen against whole cell lysate, allowing for use of this enzyme for protein-conjugated ADPr site identification by mass spectrometry [5].

While the pipeline presented here is an effective method for isolating SVP from snake venom we believe the complexity of the purification scheme, along with the lot-to-lot variability observed from commercial sources which serve as the input for this purification (data not shown), could be greatly improved upon by the availability of a recombinant, stable enzyme which could be reliably expressed, purified and scaled to meet the often large material demands of proteomic pipelines. For this reason we went on to compare the activity of SVP with that of relatively small, stable and well characterized Nudix hydrolases, which we hypothesized could cleave protein-conjugated ADPr with similar specificity.

Nudix ADPrases do not hydrolyze protein or PAR conjugated ADPr

Nudix ADPrases are responsible for the breakdown of free ADPr into its phosphoribose and adenosine monophosphate subunits, thus modulating the levels of free ADPr. This knowledge lead us to first test a group of Nudix ADPrases for hydrolase activity against protein-conjugated MAR and PAR: PARP1, an enzyme known to autoPARylate in the presence of NAD⁺, was exposed to ³²P-labeled NAD⁺ producing either ³²P-labeled PARylated (on WT PARP1) or MARylated (on the catalytically deficient PARP1 E988Q mutant) proteins to serve as substrates for hydrolysis by candidate Nudix enzymes or the positive control, SVP (Fig. 3-2a-c). From a structural perspective, the lack of activity towards ADPr could be explained by the dimeric structure of ADPrases, where each dimer is formed by monomers of an N-terminal β -sheet domain and a C-terminal Nudix domain (Fig 3-2d-g). The N-terminal domains are swapped, creating two active sites where both monomers contribute to substrate recognition (for one active site the N-terminal β -sheet of one monomer and the C-terminal Nudix domain of the opposite monomer). As shown in Fig 3-2h, ADPr is nested in the active site of ADPrase (also known as *Ec*NudF) so that the 1'-hydroxyl of the terminal ribose group is completely buried by the protein dimer (white arrowhead), preventing

conjugation to another ADPr group (or a protein). This explanation could likely be extended to the other three nucleoside sugar hydrolases tested in this study as they display the same quaternary arrangement and have a high structural homology with a pairwise root mean square deviation ranging from 0.9 to 2.0 Å (Fig 3-2d-g).

Monomeric Nudix hydrolases are capable of hydrolyzing protein and PAR conjugated ADP-ribose

In order to consider Nudix enzymes with active sites more open to fit the target ADPr group bound to either a PAR polymer or protein, we turned to Nudix families which are not swapped dimers and lack N- or C-terminal domain insertions, hypothesizing that enzymes with just the Nudix fold would have a more open active site. We chose four Nudix enzymes known to be monomeric by gel filtration, two of them (*Dr1184/CoAse* from *Deinococcus radiodurans*, Fig. 3-3c and *EcRppH* from *Escherichia coli*, Fig. 3d) degraded the ³²P-labeled PAR on the model protein PARP1 (Fig. 3-3a), while only *EcRppH* showed slight activity against ³²P-labeled MAR (Fig. 3-3b). Structural analysis and modeling of *EcRppH* revealed that the active site within these enzymes could accommodate protein conjugated ADPr, as opposed to the ADPrases reported in the previous section (Fig. 3-3e-f).

Both *HsNudT16* and *EcRppH* can degrade protein conjugated PAR and MAR to a phosphoribose tag for mass spectrometry

A recent study by Palazzo *et al* [23] has revealed that *HsNudT16*, a human Nudix (deoxy)inosine diphosphatase [37] which is also known to decap small nucleolar RNAs [38] as well as cytoplasmic mRNAs [39], has the ability to degrade protein conjugated ADPr. As shown in Fig. 3-4a-f *HsNudT16* has a high structural similarity to both *EcRppH* and *Dr1184* (which showed activity against protein conjugated ADPr, see Fig. 3-3a-b) and also possesses an open active site which would allow for the target ADPr to be conjugated to a protein or PAR polymer. Based on these similarities we postulated that *HsNudT16* would have similar activity against protein conjugated ADPr as was

observed for *EcRppH* and *Dr1184*. To test this ³²P-PARYlated or MARYlated PARP1 was exposed to increasing amounts of SVP, *EcRppH*, *Dr1184* or *HsNudT16*. As shown via autoradiographs in Fig. 3-4g-h both *HsNudT16* and *EcRppH* are able to remove both forms of ADPr, while *Dr1184* showed activity against protein conjugated PAR but not MAR.

To validate that *EcRppH* and *HsNudT16* are degrading ADPr down to its phosphoribose attachment site we treated wildtype PARP1 with SVP, *EcRppH* and *HsNudT16* for two hours at 37°C before digesting the proteins to peptides and subjecting them to phosphoenrichment on an IMAC matrix, as described by Daniels *et al* [5]. As shown in Fig. 3-5 all three enzymes (SVP, *HsNudT16* and *EcRppH*) degraded ADPr to phosphoribose, allowing for confident identification of modification sites by mass spectrometry.

3.5 Discussion

Mass spectrometry-based proteomics represents the gold standard for the study of post-translational modifications, and the field of ADP-ribosylation will surely benefit from increased access to the suite of proteomic tools that have been developed for other PTMs such as phosphorylation, acetylation and Ubiquitylation. The advent of tag-based approaches for identifying ADP-ribosylation sites has begun to provide access to these tools, but adoption has been relatively low due to technical difficulties which accompany the current methods. The work presented here promises to streamline an up-and-coming method for ADPr site identification: the simplification of ADPr to its phosphoribose attachment. Recombinant *HsNudT16* and *EcRppH* can be synthesized and purified from *E. coli*, allowing for low-cost, high yield production which can be performed in most proteomic laboratories. Furthermore, since the structure is known for both of these enzymes, it is possible to predict mutations and truncations which could potentially increase the enzyme's activity towards protein conjugated ADPr; for example, introducing mutations which will further open up the active site to allow larger ADP-ribosylated substrates access.

The work presented here has the potential to greatly aid in the elucidation of the ADP-ribosylated proteome by providing researchers the necessary tools for generating phosphoribose as a mass spectrometry friendly site localization tag of protein ADP-ribosylation sites.

3.6 Author Contributions

C.M.D., A.K.L.L., and S.B.G. wrote the manuscript. C.M.D. and S.B.G. prepared the figures. S.E.O. and C.M.D. performed mass spectrometry analyses. S.B.G. performed structural analyses. S.B.G. and P.T. prepared all Nudix proteins. C.M.D. prepared PARP1 and SVP proteins. C.M.D. designed and performed biochemical screens. All authors reviewed the manuscript.

3.7 Acknowledgements

The authors would like to thank Dr. John Pascal for kindly donating the human PARP-1 expression plasmid used to generate the WT and E988Q mutant proteins used in this study. Work was funded by an NIH grant R01-GM104135 (A.K.L.L.), the Safeway Research Foundation (A.K.L.L.), the Patrick C. Walsh Prostate Cancer Research Fund (A.K.L.L.), the Allegheny Health Network–Johns Hopkins Cancer Research Fund (A.K.L.L. and S.B.G.), a Joy Cappel Young Investigator Award (C.M.D.), an NCI training grant 5T32CA009110 (C.M.D.).

3.8 Figures

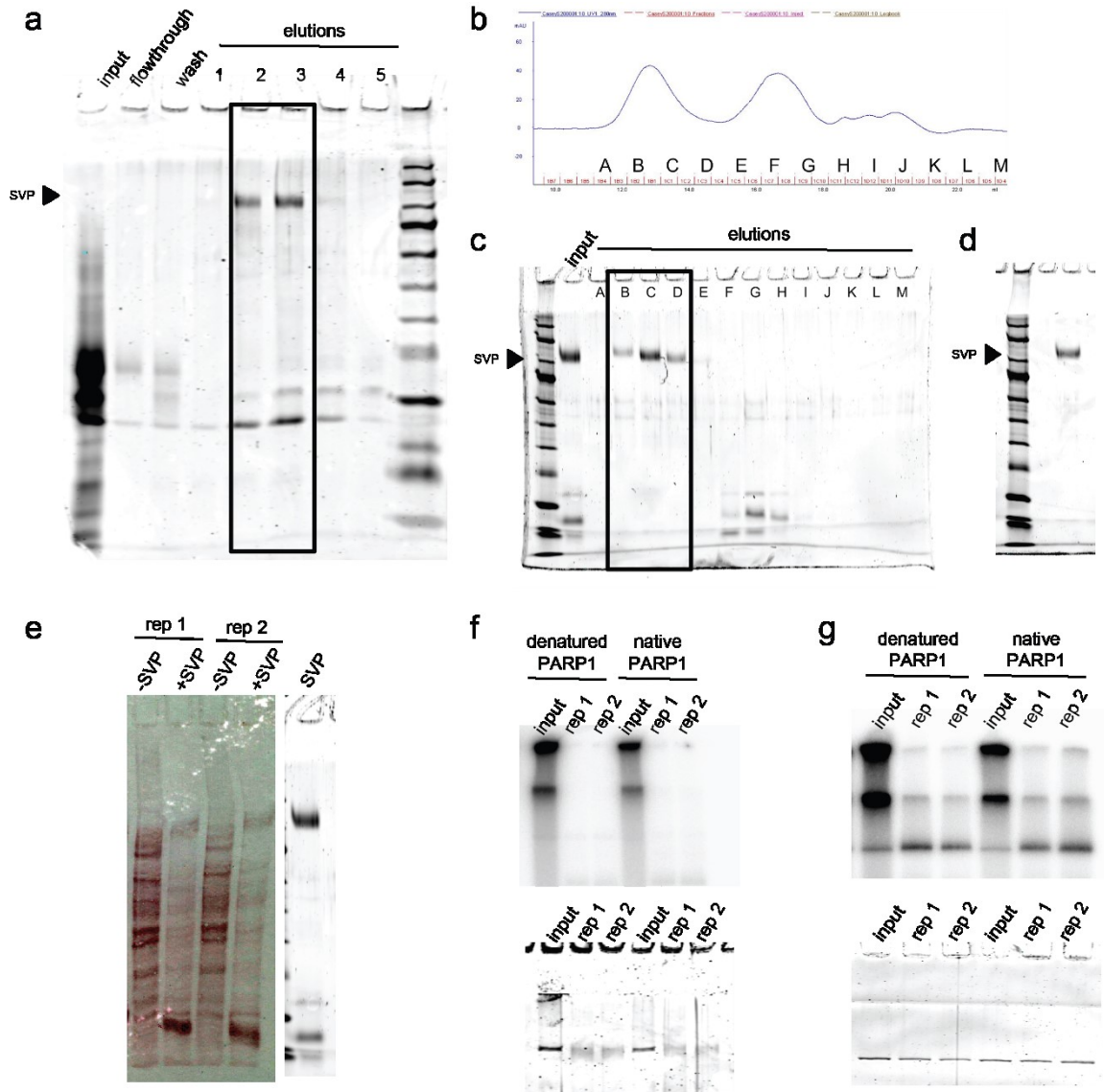


Figure 3-1 Purification of snake venom phosphodiesterase for the digestion of protein-conjugated ADP-ribose

SVP was first affinity purified on a blue sepharose column (a) followed by size exclusion chromatography (b and c), yielding a single ~110 kD band (d). Blue sepharose purification alone does not always sufficiently remove protease activity, as shown in (e) where a complex mixture of denatured proteins is mostly degraded when exposed to SVP. Panel (f) shows ^{32}P -PARylated PARP1 being degraded by the same enzyme prep used in (e) in both its native and denatured forms. Panel (g) shows the size-exclusion purified SVP degrading the ^{32}P -labeled PAR on PARP1 without any evidence of protease activity.

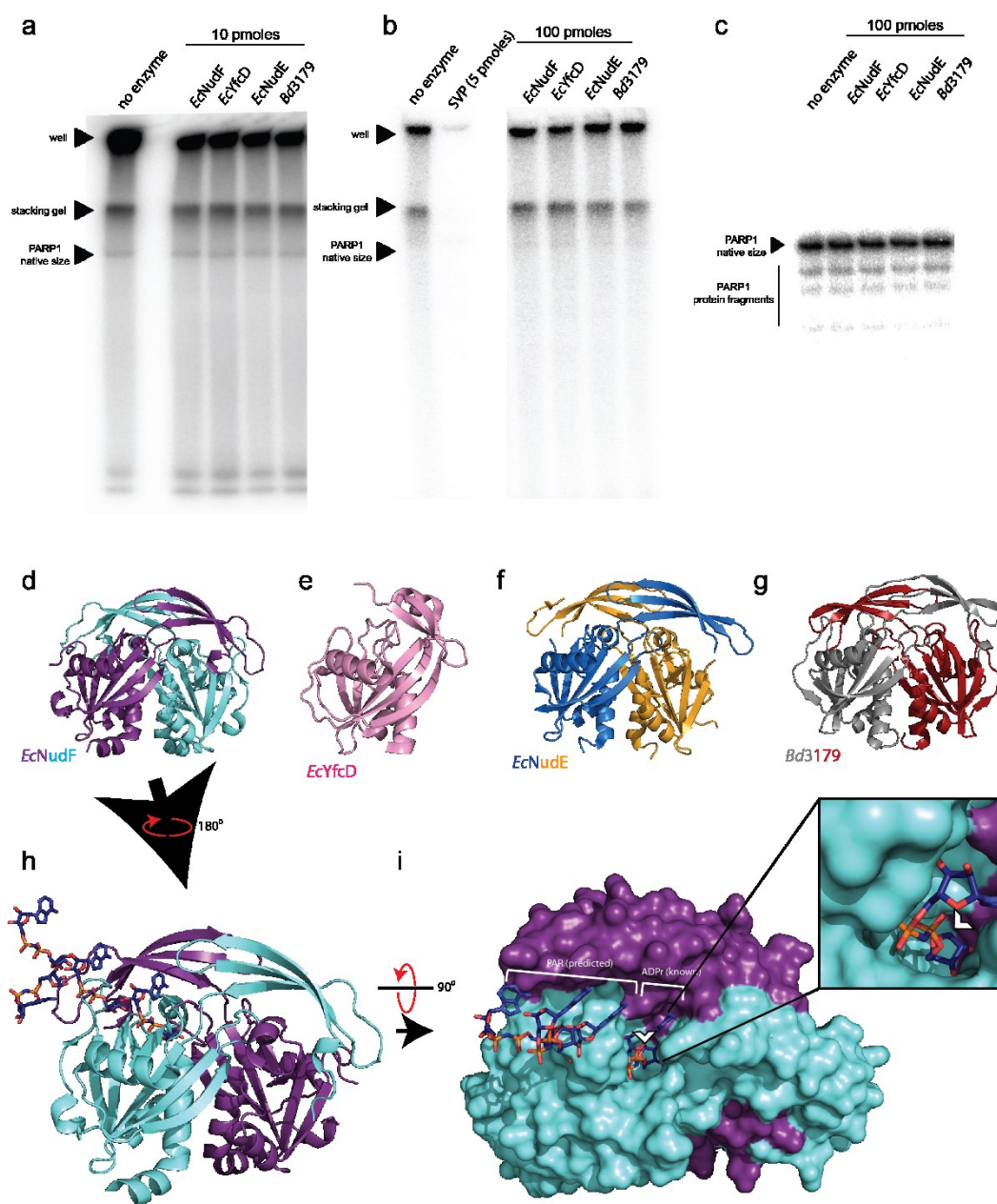


Figure 3-2 ADPrases are ineffective against protein-conjugated ADP-ribose.

(a-c) Autoradiograph showing ^{32}P -labeled mono- or poly(ADP-ribose) conjugated to PARP1 following exposure to canonical sugar hydrolases: *EcNudF*, *EcYfcD* and *EcNudE* as well as *Bd3179*. For (a) 5 pmoles of PARylated human PARP1 was exposed to 10 pmoles of hydrolase for 2 hrs at 37°C , for (b) 100 pmoles of hydrolases were utilized, while in (c) 5 pmoles of PARP1 E988Q, a mutant only capable of synthesizing mono(ADP-ribose), was exposed to 100 pmoles of hydrolases.

Ribbon diagrams show the structure of each of the enzymes used: (d) *Ea*NudF/ADPrase (PDB ID 1KHZ)[20], (e) *Ec*YfcD (PDB ID 2FKB), (f) *Ea*NudE (PDB ID 1VHG) and (g) *Bd*3179[25]. Panel (h) shows a surface representation of *Ea*NudF with modeled PAR polymer based on the complex with a nonhydrolyzable ADPr[40]. Panel (i) shows a surface representation of *Ea*NudF with the modeled PAR polymer, the inset shows how the terminal ribose is protected and buried within the enzyme. The white arrowhead indicates where protein conjugation would occur on ADPr.

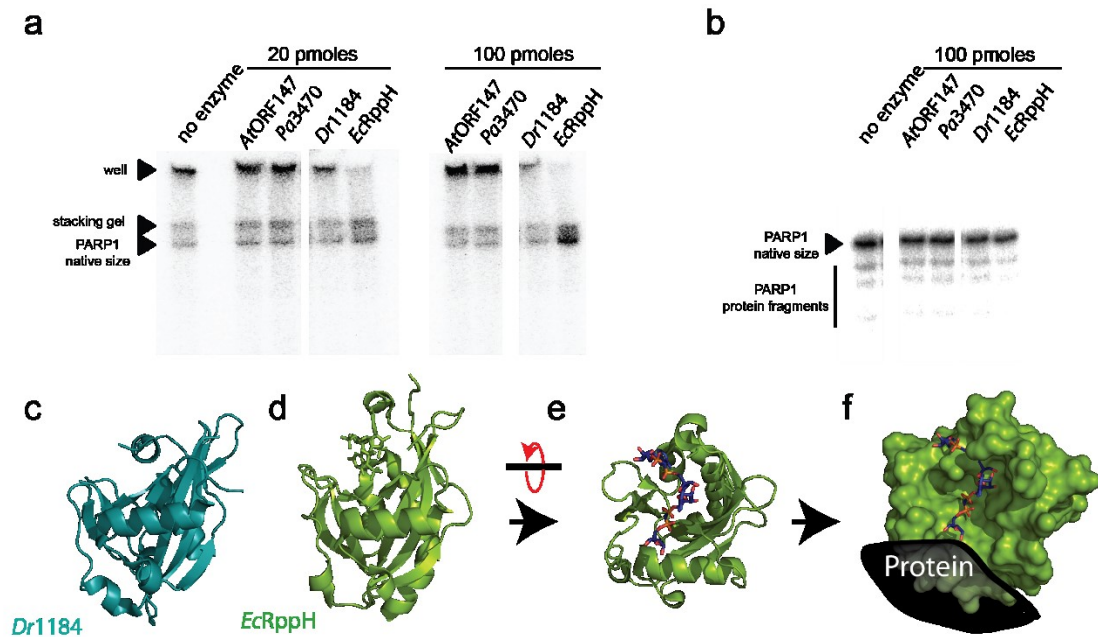


Figure 3-3 Single domain Nudix hydrolases show activity against protein-conjugated PAR.

(a,b) Autoradiograph showing the ^{32}P -labeled PAR (a) or MAR (b) conjugated to PARP1 following exposure to Nudix hydrolases: *AtORF147*, *Pa3470*, *Dr1184*, and *EcRppH*. In this assay 5 pmoles of PARylated PARP1 was exposed to either 20 or 100 pmoles of hydrolase for 2 hrs at 37°C . (c) Ribbon model of the structure of *Dr1184/CoAse* (teal, PDB ID 1NQY), (d) Ribbon model of the structure of *EcRppH* (green, PDB ID 4S2Y) with capped RNA bound (green). (e) PAR modeled into the active site of *EcRppH* based on the known orientation of RNA bound in the reported structure[41]. Panel f shows a surface representation of *EcRppH* with PAR conjugated to a protein in the binding site.

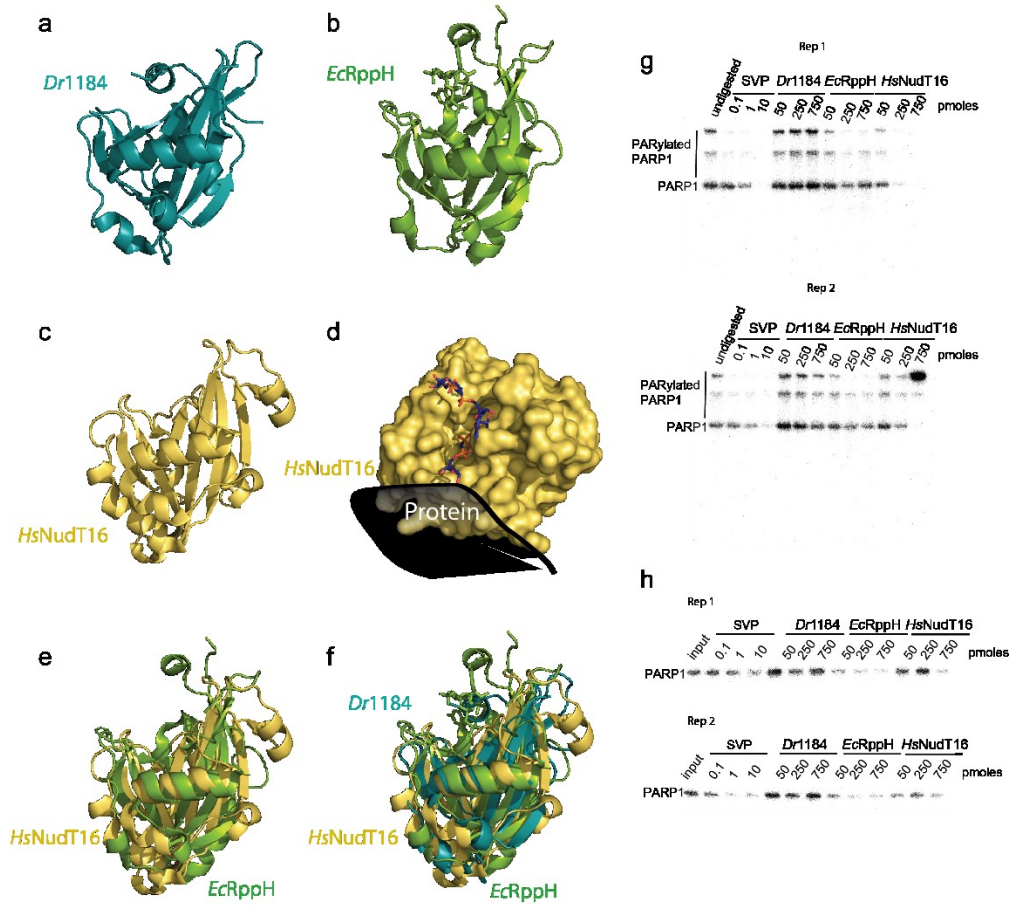


Figure 3-4 Human Nud16 degrades protein conjugated ADPr.

Panels (a) and (b) show ribbon diagrams of *Dr1184* and *EcRppH* for comparison. Panel (c) shows a ribbon diagram of *HsNudT16* (PDB ID 2xSQ), (d) PAR has been modeled into the *HsNudT16* structure with a protein depicted at the conjugation site on ADPr. Panel (e) shows an alignment between *HsNudT16* and *EcRppH*, while (f) shows an alignment of *HsNudT16*, *EcRppH* and *Dr1184*. Panels (g) and (h) show the removal of ³²P-labeled ADPr from 5 pmoles of PARylated (g) or MARylated (h) PARP1 by increasing amounts of SVP, *Dr1184*, *EcRppH* or *HsNudT16*.

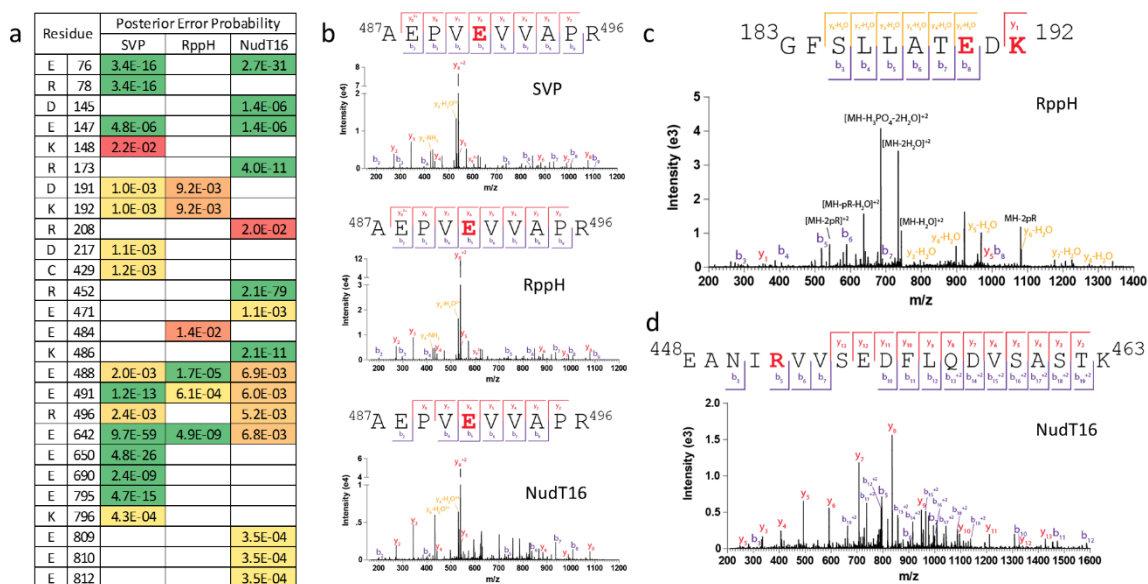


Figure 3-5 RppH and Nud16 for the generation of phosphoribose as an ADPr tag for mass spectrometry.

(a) Phosphoribosylation sites with their corresponding posterior error probabilities identified on 0.05 nmoles of the model protein PARP1 after the conversion of ADPr to phosphoribose by 0.1 nmoles of SVP, 3 nmoles of *Ec*RppH or 3 nmoles of *Hs*NudT16. (b) E491 on PARP1 was identified as a PARP1 automodification site following SVP, *Ec*RppH and *Hs*NudT16 treatment. (c) E190 and K192 were identified as PARP1 automodification sites on PARP1 following *Ec*RppH digestion. (d) Identification of R496 as a PARP1 automodification site following *Hs*NudT16 treatment.

3.9 References

1. Curtin, N.J. and C. Szabo, *Therapeutic applications of PARP inhibitors: anticancer therapy and beyond*. Mol Aspects Med, 2013. **34**(6): p. 1217-56.
2. Hottiger, M.O., et al., *Toward a unified nomenclature for mammalian ADP-ribosyltransferases*. Trends Biochem Sci, 2010. **35**(4): p. 208-19.
3. Vyas, S., et al., *Family-wide analysis of poly(ADP-ribose) polymerase activity*. Nat Commun, 2014. **5**: p. 4426.
4. Daniels, C.M., S.E. Ong, and A.K. Leung, *The Promise of Proteomics for the Study of ADP-Ribosylation*. Mol Cell, 2015. **58**(6): p. 911-24.
5. Daniels, C.M., S.E. Ong, and A.K. Leung, *A phosphoproteomic approach to characterize protein mono and poly(ADP-ribosyl)ation sites from whole cell lysate*. J Proteome Res, 2014.
6. Chapman, J.D., et al., *Mapping PARP-1 auto-ADP-ribosylation sites by liquid chromatography-tandem mass spectrometry*. J Proteome Res, 2013.
7. Bessman, M.J., D.N. Frick, and S.F. O'Handley, *The MutT proteins or "Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes*. J Biol Chem, 1996. **271**(41): p. 25059-62.
8. Mildvan, A.S., et al., *Structures and mechanisms of Nudix hydrolases*. Arch Biochem Biophys, 2005. **433**(1): p. 129-43.
9. Tong, L., S. Lee, and J.M. Denu, *Hydrolase regulates NAD⁺ metabolites and modulates cellular redox*. J Biol Chem, 2009. **284**(17): p. 11256-66.
10. Perraud, A.L., et al., *ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology*. Nature, 2001. **411**(6837): p. 595-9.
11. Durnin, L., et al., *Adenosine 5-diphosphate-ribose is a neural regulator in primate and murine large intestine along with beta-NAD(+)*. J Physiol, 2012. **590**(Pt 8): p. 1921-41.
12. Jacobson, E.L., D. Cervantes-Laurean, and M.K. Jacobson, *Glycation of proteins by ADP-ribose*. Mol Cell Biochem, 1994. **138**(1-2): p. 207-12.
13. Miwa, M. and T. Sugimura, *Splitting of the ribose-ribose linkage of poly(adenosine diphosphate-ribose) by a calf thymus extract*. J Biol Chem, 1971. **246**(20): p. 6362-4.
14. Steffen, J.D. and J.M. Pascal, *New players to the field of ADP-ribosylation make the final cut*. EMBO J, 2013. **32**(9): p. 1205-7.
15. Shull, N.P., S.L. Spinelli, and E.M. Phizicky, *A highly specific phosphatase that acts on ADP-ribose 1''-phosphate, a metabolite of tRNA splicing in Saccharomyces cerevisiae*. Nucleic Acids Res, 2005. **33**(2): p. 650-60.
16. Dolle, C., J.G. Rack, and M. Ziegler, *NAD and ADP-ribose metabolism in mitochondria*. FEBS J, 2013. **280**(15): p. 3530-41.

17. Peterson, F.C., et al., *Orphan macrodomain protein (human C6orf130) is an O-acyl-ADP-ribose deacylase: solution structure and catalytic properties*. J Biol Chem, 2011. **286**(41): p. 35955-65.
18. Kim, H., E.L. Jacobson, and M.K. Jacobson, *Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases*. Science, 1993. **261**(5126): p. 1330-3.
19. McLennan, A.G., *The Nudix hydrolase superfamily*. Cell Mol Life Sci, 2006. **63**(2): p. 123-43.
20. Gabelli, S.B., et al., *The structure of ADP-ribose pyrophosphatase reveals the structural basis for the versatility of the Nudix family*. Nat Struct Biol, 2001. **8**(5): p. 467-72.
21. Deana, A., H. Celesnik, and J.G. Belasco, *The bacterial enzyme RppH triggers messenger RNA degradation by 5' pyrophosphate removal*. Nature, 2008. **451**(7176): p. 355-8.
22. Li, Y., M. Song, and M. Kiledjian, *Differential utilization of decapping enzymes in mammalian mRNA decay pathways*. RNA, 2011. **17**(3): p. 419-28.
23. Palazzo, L., et al., *Processing of Protein ADP-ribosylation by Nudix Hydrolases*. Biochem J, 2015.
24. O'Handley, S.F., et al., *Orf186 represents a new member of the Nudix hydrolases, active on adenosine(5')triphospho(5')adenosine, ADP-ribose, and NADH*. J Biol Chem, 1998. **273**(6): p. 3192-7.
25. Wolff, K.A., et al., *A redox regulatory system critical for mycobacterial survival in macrophages and biofilm development*. PLoS Pathog, 2015. **11**(4): p. e1004839.
26. Kang, L.W., et al., *Structure of a coenzyme A pyrophosphatase from Deinococcus radiodurans: a member of the Nudix family*. J Bacteriol, 2003. **185**(14): p. 4110-8.
27. Bessman, M.J., et al., *The gene ygdP, associated with the invasiveness of Escherichia coli K1, designates a Nudix hydrolase, Orf176, active on adenosine (5')-pentaphospho-(5')-adenosine (Ap5A)*. J Biol Chem, 2001. **276**(41): p. 37834-8.
28. Xu, W., et al., *A new subfamily of the Nudix hydrolase superfamily active on 5-methyl-UTP (ribo-TTP) and UTP*. J Biol Chem, 2003. **278**(39): p. 37492-6.
29. L. Tresaugues, M.M., C.H. Arrowsmith, H. Berglund, R. D. Busam, R. Collins, L.G.Dahlgren, A. Edwards, S. Flodin, A. Flores, S. Graslund, M. Hammarstrom, A. Johansson, I. Johansson, A. Kallas, T. Karlberg, T. Kotenyova, L. Lehtio, M.E. Nilsson, T. Nyman, C. Persson, J. Sagemark, H. Schueler, L. Svensson, A.G. Thorsell, S. Van Den Berg, M. Welin, J. Weigelt, M. Wikstrom, P. Nordlund, *Human Nudix motif 16 (NUDT16)*. <http://www.thesgc.org/structures/3cou>.

30. Schrodinger, LLC, *The PyMOL Molecular Graphics System, Version 1.3r1*, 2010.
31. Nishizuka, Y., et al., *Studies on the polymer of adenosine diphosphate ribose. I. Enzymic formation from nicotinamide adenine dinucleotide in mammalian nuclei*. J Biol Chem, 1967. **242**(13): p. 3164-71.
32. Kawaichi, M., K. Ueda, and O. Hayaishi, *Multiple autopoly(ADP-ribosyl)ation of rat liver poly(ADP-ribose) synthetase. Mode of modification and properties of automodified synthetase*. J Biol Chem, 1981. **256**(18): p. 9483-9.
33. Desmarais, Y., et al., *Enzymological properties of poly(ADP-ribose)polymerase: characterization of automodification sites and NADase activity*. Biochim Biophys Acta, 1991. **1078**(2): p. 179-86.
34. Rokyta, D.R., et al., *The venom-gland transcriptome of the eastern diamondback rattlesnake (Crotalus adamanteus)*. BMC Genomics, 2012. **13**: p. 312.
35. Oka, J., K. Ueda, and O. Hayaishi, *Snake venom phosphodiesterase: simple purification with Blue Sepharose and its application to poly(ADP-ribose) study*. Biochem Biophys Res Commun, 1978. **80**(4): p. 841-8.
36. Hengel, S.M. and D.R. Goodlett, *A Review of Tandem Mass Spectrometry Characterization of Adenosine Diphosphate-Ribosylated Peptides*. Int J Mass Spectrom, 2012. **312**: p. 114-121.
37. Abolhassani, N., et al., *NUDT16 and ITPA play a dual protective role in maintaining chromosome stability and cell growth by eliminating dIDP/IDP and dITP/ITP from nucleotide pools in mammals*. Nucleic Acids Res, 2010. **38**(9): p. 2891-903.
38. Ghosh, T., et al., *Xenopus U8 snoRNA binding protein is a conserved nuclear decapping enzyme*. Mol Cell, 2004. **13**(6): p. 817-28.
39. Song, M.G., Y. Li, and M. Kiledjian, *Multiple mRNA decapping enzymes in mammalian cells*. Mol Cell, 2010. **40**(3): p. 423-32.
40. Gabelli, S.B., et al., *Mechanism of the Escherichia coli ADP-ribose pyrophosphatase, a Nudix hydrolase*. Biochemistry, 2002. **41**(30): p. 9279-85.
41. Vasilyev, N. and A. Serganov, *Structures of RNA Complexes with the Escherichia coli RNA Pyrophosphohydrolase RppH Unveil the Basis for Specific 5'-End-dependent mRNA Decay*. J Biol Chem, 2015. **290**(15): p. 9487-99.

4 The Promise of Proteomics for the Study of ADP-ribosylation

Casey M. Daniels¹, Shao-En Ong², and Anthony K.L. Leung¹

¹Department of Biochemistry and Molecular Biology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205, United States

²Department of Pharmacology, University of Washington, Seattle, Washington 98195, United States

Published as

Casey M. Daniels, Shao-En Ong, Anthony K.L. Leung, The Promise of Proteomics for the Study of ADP-Ribosylation, *Molecular Cell*, Volume 58, Issue 6, 18 June 2015, Pages 911-924, ISSN 1097-2765, <http://dx.doi.org/10.1016/j.molcel.2015.06.012>.
(<http://www.sciencedirect.com/science/article/pii/S1097276515004517>)

4.1 Abstract

ADP-ribosylation is a post-translational modification where single units (mono-ADP-ribosylation) or polymeric chains (poly-ADP-ribosylation) of ADP-ribose are conjugated to proteins by ADP-ribosyltransferases. This post-translational modification and the ADP-ribosyltransferases (also known as PARPs) responsible for its synthesis have been found to play a role in nearly all major cellular processes, including DNA repair, transcription, translation, cell signaling and cell death. Furthermore, dysregulation of ADP-ribosylation has been linked to diseases including cancers, diabetes, neurodegenerative disorders and heart failure, leading to the development of therapeutic PARP inhibitors, many of which are currently in clinical trials. The study of this therapeutically important modification has recently been bolstered by the application of mass spectrometry-based proteomics, arguably the most powerful tool for the unbiased analysis of protein modifications. Unfortunately, progress has been hampered by the inherent challenges that stem from the physicochemical properties of ADP-ribose which as a post-translational modification is highly charged, heterogeneous (linear or branched polymers, as well as monomers), labile, and found on a wide range of amino acid acceptors. In this perspective, we discuss the progress that has been made in addressing these challenges, including the recent breakthroughs in proteomics techniques to identify ADP-ribosylation sites, and future developments to provide a proteome-wide view of the many cellular processes regulated by ADP-ribosylation.

4.2 Introduction

ADP-ribosylation refers to the transfer of the ADP-ribose group from NAD^+ to target proteins post-translationally. This post-translational modification (PTM) can be added on to amino acids of diverse chemistry, including aspartate, glutamate, lysine, arginine and cysteine. ADP-ribose groups can be attached singly as mono(ADP-ribose) (MAR) or in polymeric chains as poly(ADP-ribose) (PAR) by the enzymatically active members of the family of 17 human ADP-ribosyltransferases (ARTs), commonly known as poly(ADP-ribose) polymerase (PARPs) [1, 2]. Together, MAR and PAR regulate fundamental cellular processes through their roles as signaling

molecules [3, 4] and post-translational modifications [5-7]. In addition, ADP-ribosylation has been shown to be a therapeutically important modification in cancers, neurodegenerative diseases, ischemia and inflammatory disorders [8], where PARPs are hotly pursued drug targets by pharmaceutical companies [9]. Over a hundred clinical trials for the treatment of cancers have been carried out for PARP1 inhibitors and many ongoing trials are in late stages [10, 11]. Notably, these anti-cancer drugs can also cross-react with other PARPs [12], which are increasingly appreciated for their multifaceted roles in the cell (Figure 4-1) [6, 13]. Identifying the substrate specificities of these PARPs will help elucidate distinct functions of this 17-member family and may have therapeutic implications in designing PARP inhibitor-based therapies. Recent advances in mass spectrometry (MS)-based methods for characterizing ADP-ribosylated proteins have opened up unprecedented possibilities to explore the functions of this family of enzymes and provide insights into the clinical relevance of this under-studied protein modification.

MS-based proteomics offers three types of data that genomics and transcriptomics cannot: protein-protein interaction mapping (interactomics), identification of protein modification sites, and quantitative information at the protein level (for an in depth overview of the potential held by MS-based proteomics we recommend [14]). A complete map of the ADP-ribosylated proteome will include all three elements, providing insights into how ADP-ribosylated substrates are regulated via recruitment of MAR/PAR-binding proteins, their sites of modification, and abundance in cells. While the ADP-ribosylated interactome has been explored in the last decade, it is only recently that MS-based techniques have been available for the identification of ADP-ribosylated sites at the proteome scale. In this perspective we will explore how MS-based proteomics can help address several important questions in the field of ADP-ribosylation: (1) What is the significance of the many potential amino acid attachment sites? Which attachments are regulated by which enzymes? (2) How can we distinguish between sites of MAR and PAR, and between the many possible structures of PAR, including length and branch variants? How important are these distinctions? (3) What does an increase in cellular PARylation levels mean? Does it reflect an increase in the number of amino acid

site modifications, an increase in the number of ADP-ribose units at existing sites, or an increase in unconjugated PAR levels? (4) Are all ADP-ribosylation sites physiologically significant? In the following sections, we will discuss the inherent challenges, existing solutions and future needs to address these critical questions for a complete, functional understanding of the ADP-ribosylated proteome.

4.3 Investigating the ADP-ribosylated Proteome by Mass Spectrometry: Challenges

Mapping of MARylated and PARylated (collectively, ADP-ribosylated) proteomes requires robust protocols to overcome the dynamic, heterogeneous and labile nature of these modifications. An initial challenge is the variable PAR attachment sites, which can be found on acidic and basic residues, a list that expands when MARylation sites are also considered (see later sections). This variability results in a wide range of chemical and enzymatic sensitivities [15], greatly hindering the identification of an intact, complete ADP-ribosylated proteome. Secondly, the modification itself is typically found at low levels in cells and exhibits very fast attachment/removal kinetics [16, 17], making robust enrichment methods a critical component for elucidating the ADP-ribosylated proteome. Thirdly, the structure of the PAR polymer poses a practical challenge, as it is heterogeneous (between 2 and 200 subunits *in vivo*, can be branched or linear [18]) and highly charged, characteristics incompatible with most MS methods. Here, we will consider the methods that have addressed and overcome subsets of these challenges and the potential for further progress on those that remain.

4.4 A Draft of the ADP-ribosylated Interactome

Molecular interactions can serve as an early indicator of molecular functions, and a sizeable contribution has already been made to the field of ADP-ribosylation by several large scale proteomics studies that identify proteins associated with MAR and/or PAR which are summarized in Figure 4-2 panel A [19-22]. These studies used a common experimental design: human cells were exposed to DNA damaging agents, a classical stimulant of PARP-1 PARylation activity, before being lysed and

subjected to enrichment of ADP-ribosylated proteins, followed by MS-based protein identification. Because the enrichment is performed under a range of non-denaturing conditions in all of these studies, the proteins identified include not only ADP-ribosylated proteins but also ADP-ribose binding proteins and the larger non-covalent interaction networks, thereby providing an aggregate picture of the ADP-ribosylated interactome. Using all 832 proteins identified in these studies (Supplementary Table 4-2), a draft map of biological processes enriched in the DNA damage-induced ADP-ribosylated interactome is presented in Figure 4-2 panel B and Supplementary Figure 1 in detail. While the DNA damage response is the canonical role for PARylation in cells, it is clear that additional roles for ADP-ribosylation are present even following genomic insult. In particular, there is a significant enrichment of RNA processing factors (purple boxes), a trend which was noted individually by each group. Such enrichment may be linked to the similarity of the chemical and electrostatic properties of PAR and RNA — cellular biopolymers that are able to share binding partners (e.g. [23]). Another noted enrichment is seen for cellular macromolecular complex assembly, exemplified in mitotic spindles [24], nucleoli [25], stress granules [26], DNA repair complexes [27], and nuclear matrices [28], possibly owing to the polymeric nature of PAR and the plethora of PAR binding domains which may target this polymer as a structural scaffold. Such proteome-wide views of the biological processes regulated by ADP-ribosylation sends researchers and clinicians a key message: a reduction in ADP-ribosylation by PARP inhibitors impacts many aspects of cellular function and should not be seen as a simple block to DNA repair.

In light of the similarities in experimental design, the methods chosen for cell lysis and enrichment have proven to be critical determinants of the interactome observed by each group. Variations in the enrichment method for ADP-ribosylated proteins produced two nearly distinct sets of proteins (see Figure 4-2 panel C), partly resulting from biased affinity of the 10H antibody for PAR polymers longer than 20 subunits [29] while the Af1521 macrodomain enriches for both MARYlated and PARYlated proteins [30]. Such biased affinity may help explain why the Af1521 macrodomain-enriched interactome contains more known ADP-ribosylated substrates (as

determined by their inclusion in site identification studies [31, 32]) than the 10H antibody-enriched interactome (see Supplementary Figure 4-2), as the longer polymers targeted by the antibody may serve as baits for PAR binding proteins and their interactors. The 10H-derived interactome can be separated into unique networks based lysis buffer composition (Figure 4-2 panel C), supporting that many of these protein-protein and protein-PAR interactors are non-covalent and subject to charge disruption. Of note, Nielsen and co-workers emphasized the inclusion of PARP inhibitors in cell lysis buffer to prevent the DNA sheared during the cell lysis procedure from activating the DNA damage-responsive PARPs *in vitro*; prevention of this activation cuts down on non-physiological PAR-dependent interactions formed in cell lysate [21], an observation that may further explain the unique identifications in the studies shown in Figure 4-2 panel C. While these pioneering studies highlight the importance for the consideration of lysis conditions and enrichment methods, it is clear that we have yet to approach saturation in probing the complete ADP-ribosylated interactome; we expect that a more complete interactome will be obtained using complementary strategies to induce and enrich ADP-ribose. Besides DNA damage, it is equally important to characterize the ADP-ribosylated interactomes under other cellular stress as well as the interactomes within various PAR-enriched cellular macromolecular complexes. Healthy, unstressed cells have also been shown to maintain low basal levels of PAR, with cellular PARylation patterns distinct at different stages of the cell cycle and in different cellular compartments [13]. Though it is quite common to increase the amount of endogenous ADP-ribosylated substrates by long-term knockdown of the PAR degradative enzyme PARG, such treatment will likely cause non-physiological changes (as shown in the PARG¹¹⁰ knockout mouse [33, 34]). While the recent development of cell-permeable PARG inhibitors may provide an alternative to increase the amount of substrates without requiring long-term treatment [35], it is a priority to improve the existing methods for enriching ADP-ribosylated substrates (reviewed in [36]) and increase the sensitivity of MS to detect them from native cell conditions.

By definition, the ADP-ribosylated interactome is composed of covalently ADP-ribosylated substrates, ADP-ribose binding proteins, and their interacting proteins. With the ability to synthesize

MAR or PAR with a defined number of ADP-ribose groups [37], it is foreseeable to further refine the mapping of the proteome that binds to single or multiple ADP-ribose groups non-covalently. Parallel development of techniques to identify the attachment sites of ADP-ribosylation has already allowed for definitive identification of ADP-ribosylated substrates at the proteome level ([31, 32]; see the next section)). Combination of these complementary sets of proteomic data will allow researchers more precision in mapping the connections within the ADP-ribosylated interactome.

4.5 Characterizing ADP-ribosylation at the Level of the Amino Acid Attachment Sites

While MARYlation and PARylation have long been considered two classes of PTMs, it is useful, and perhaps more accurate, to consider their attachment sites together as a single modification. The first reason for this consideration stems from knowledge of the PAR degradative enzyme PARG [38], which is capable of transforming PARylated substrates into MARYlated ones, effectively blurring the lines between sites of mono and poly(ADP-ribose). Secondly, there is evidence of cooperative efforts between enzymes capable of adding mono and poly(ADP-ribose) to proteins [39] which may result in a PARP adding polymer to an existing MAR initiation site — an occurrence which has also been shown *in vitro* through PARP-1 elongation of MARYlated agarose beads [40]. This notion of shared sites for MAR and PAR synthesis is taken further by the demonstration that PARP-4 exhibits MARYlating activity in isolation but has PARylating activity in its native vault protein complex; this change in activity presumably arises through cooperation with other members of the complex, none of which are known PARPs [1, 41]. For these reasons, characterization of ADP-ribosylation attachment sites remains distinct from characterization of the heterogeneous molecule (mono/poly, linear/branched) that occupies these sites. Accordingly, the MS-based methods for ADP-ribosylation site identification discussed in this section are restricted to identifying the site of the PTM attachment following removal of any subunits beyond the protein-proximal monomer, offering no information with respect to the original size or structure of the corresponding PTM.

A major analytical challenge in identifying ADP-ribosylation attachment sites comes from the wide variety of amino acids that can be ADP-ribosylated, including glutamic and aspartic acids, serines, threonines [42], phosphoserines [43], cysteines [44], asparagines [45], arginines [46], lysines [47] and diphthamides [48]. This large collection of ADP-ribose acceptors provides a number of unique attachment structures (Figure 4-3) that differ in chemical and enzymatic sensitivities, e.g. acidic, but not basic, amino acids lose ADP-ribose in the presence of high pH, hydroxylamine quickly releases ADP-ribose groups from modified glutamate, asparatate and, less readily, from arginine, and ADP-ribose is exclusively removed from arginine in the presence of the ADPr hydrolase ARH1 [15, 49, 50]. Though the majority of ADP-ribosylated sites are sensitive to hydroxylamine [51], hydroxylamine-insensitive sites, such as lysine, may also serve important biological roles [52]. Phosphorylated tyrosine sites are relatively rare in comparison to phosphoserine and phosphothreonine, yet they play indispensable roles in cellular biology [53]; it would be important, therefore, to study all intracellular protein residue–ADP-ribose attachments to understand the significance of each site of modification.

Several sample preparation methods have been developed to study ADP-ribosylation sites by MS. The first relies on the unambiguous identification of MArYlated sites, which can be identified as a 541.06 Dalton mass shift above the unmodified form of the peptide (Figures 4-4 panels A and B). A distinct advantage of this method comes from the reliable fragmentation of the modification itself during standard peptide fragmentation, providing diagnostic ions that can confirm the ADP-ribosylation state of the modified peptide [54-56]. This method has also been utilized for the identification of PARylation sites following treatment of defined substrates *in vitro* with PARG or ARH3, both of which leave MAR at the otherwise mass variant PAR attachment site [57, 58]. It should be noted, however, that an inherent uncertainty underlies a subset of site identifications following PARG/ARH3 treatment as these enzymes release free ADP-ribose — a molecule that has been shown to spontaneously ADP-ribosylate the N-terminus of proteins and peptides as well as lysine and cysteine residues [44, 59, 60]. As such modifications have the potential to form in any

environment rich in free ADP-ribose (e.g. in the vicinity of PARG/ARH3 digestion), the occurrence and significances of these non-enzymatic modifications in cells remain important unanswered questions (see Supplementary Text for further discussion). As such, their presence cannot currently be attributed exclusively to either sample preparation or intracellular biology, particularly when PARG (or any enzyme capable of producing free ADP-ribose) is present in both scenarios, and the field would greatly benefit from performing a series of experiments to clearly establish or dispel whether non-enzymatic ADP-ribosylation should be a concern for proteomics studies.

Two alternatives have been demonstrated in recent studies to identify ADP-ribosylation sites at the proteome level. The first, digestion of MAR and PAR down to their phosphoribose attachment sites (Figures 4A and 4C) [32, 61, 62], relies upon the pyrophosphatase activity of snake venom phosphodiesterase [63], a standard enzyme for *in vitro* PAR digestion [64]. Similar to the PARG/ARH3 method, the chemistry of the attachment site is maintained, however the iso-ADP-ribose fragments released by phosphodiesterase do not allow for formation of the reactive aldehyde group which has shown to be responsible for spontaneous ADP-ribosylation [60]. The apparent unbiased digestion of PAR and MAR by SVP suggests that this method will be amenable to all forms of amino acid attachments, and has indeed produced acidic, basic and nucleophilic site identifications from endogenously modified proteins [1, 32]. The second method relies upon the release of ADP-ribose from acidic (glutamic and aspartic) amino acid residues by hydroxylamine, a standard method for distinguishing between amino acid acceptors of ADP-ribose [15]. The utility of this method lies in the alteration of the acidic group following hydroxylamine release of ADP-ribose (Figure 4A); the resultant hydroxamic acid derivative produces a mass shift of 15.01 Daltons, which is easily distinguishable by MS (Figure 4D) [31]. Though limited to identifying only acidic ADP-ribosylation sites, this method has provided a list of 1,048 sites on 340 proteins from the acidic ADP-ribosylated proteome, highlighting for the first time the widespread modification of substrate proteins in cells [31].

With the ability to definitively identify ADP-ribosylation sites, it is now possible to begin addressing the roles of protein ADP-ribosylation. The functional impact of such modified sites can, to some extent, be addressed by mutagenesis studies using recombinant proteins or by targeted genome editing techniques in cells. However, unique difficulties accompany these classic means of characterizing PTM effects, as point mutations are limited by the large number of amino acids that can be ADP-ribosylated (Figure 4-3). For example, mutation of a glutamic acid to an aspartic acid will not guarantee a lack of ADP-ribosylation, requiring researchers to, in the interest of blocking ADP-ribosylation, mutate acidic sites to non-acidic residues. The requirement of such mutagenesis strategies further complicates the interpretation of molecular or cellular effects — is ADP-ribosylation of the residue important, or has the loss of an acidic residue changed the structure or interaction network of the protein? As an alternative to blocking ADP-ribosylation by mutational means, chemical strategies have been developed to introduce ADP-ribose groups at specific residues on purified peptides [65-67]; this technique could allow researchers to mimic the ADP-ribosylated form of a protein by conjugating the modified peptide of interest to the terminus/termini of the parent protein, a technique (termed semisynthesis) which has allowed for functional analysis of phosphorylated proteins in vitro [68]. Another way to ascertain functional roles of these sites involves following their modification status temporally upon treatment that induces or inhibits ADP-ribosylation. For example, quantitative proteomics techniques have already been utilized to map out the temporal coordination of ADP-ribose related protein complexes in response to DNA damage, a necessary step toward understanding the mechanism of ADP-ribose dependent DNA damage repair [20, 22]. With these newly developed site identification techniques we can further define the temporal changes of the ADP-ribosylated substrates at the site level, potentially indicating which particular sites are of physiological significance. Additionally, it has been shown that only a subset of ADP-ribosylation sites within the proteome are sensitive to treatment by chemotherapeutic PARP inhibitors currently in Phase III clinical trials [31]. These variable responses to PARP inhibition may

indicate the mechanism of action of these drugs, providing the molecular basis of the clinical benefits and side effects observed in patients.

4.6 Defining Target Specificity for Addition and Removal of ADP-ribosylation

Given the large number of cellular processes regulated by PARPs (see Figure 1), it will be interesting to determine the shared and unique substrates of each of the enzymatically-active family members. Using a protein microarray that consists of 8,000 proteins, two groups have identified the sub-proteomes that can be modified by PARP-2, PARP-10 and PARP-14 *in vitro* [69, 70].

Alternatively, the Cohen group has engineered PARP-1 and PARP-2 mutants that specifically use a bio-orthogonal NAD⁺ analogue for the identification of their respective PARP-specific substrates from nuclear extracts *in vitro* [71]. The majority of proteins modified by individual PARPs are distinct, suggesting that each PARP exhibits unique substrate specificity (Figure 4-5, Supplementary Table 4-3). When coupled with site identification techniques, it is now possible to determine if there is a defined motif surrounding the ADP-ribosylation sites modified by each PARP. For example, whether any of the PARPs are responsible for the consensus sequence of PXE*, E*P, PXXE or E*XXG surrounding the modified glutamate (E*) residue, as identified by the Yu group recently [31]. Similar experimental designs may allow us to deduce whether there are specific motifs for modification by individual PARPs, such as those identified for PARP-5a substrates [72].

One puzzling piece of data from the current studies is that ADP-ribosylation sites auto-modified by each PARP are found at diverse amino acids, such as acidic (Glu/Asp), basic (Lys, Arg) and nucleophilic (Cys) residues; this apparent lack of specificity is true for PARPs that add multiple (PARP-1) or single ADP-ribose groups (PARP-3, PARP-6, PARP-10, PARP-11, PARP-12 and PARP-16) [1, 32]. This flexibility in amino-acid acceptor residues argues against the amino acid specificity of these enzymes, at least during *in vitro* auto-modification. One possible explanation is that these PARPs are acting as NADases, which hydrolyze NAD⁺ *in vitro* [73], and the released ADP-ribose groups chemically conjugate to reactive amino acid residues. Though no studies have yet

to investigate such non-enzymatic modification on PARPs, Cervantes-Laurean *et al* showed that histones can be modified non-enzymatically by incubation with ADP-ribose *in vitro* and deduced that lysines are the primary sites [60]. On the other hand, only cysteine residues were identified in auto-modified PARP-8 *in vitro*, suggesting that certain PARPs may have defined amino acid specificity [1]. It will therefore be of interest to examine whether there are any amino acid preferences on endogenous protein substrates of each PARP at a proteome-wide scale. One major drawback of the current techniques to identify proteome-wide enzyme–substrate relationships is that these experiments were all performed *in vitro*, thus losing the proper physiological context (e.g., cellular localization, enzyme concentration, protein modification states). Therefore, techniques are urgently needed to identify PARP-specific proteomes in cells.

So far, hydrolases that remove the single ADP-ribose groups from arginine and glutamate have been identified (Table 4-1), but it is not clear whether modifications at other amino acids are reversible. Do hydrolases exhibit amino acid specificity with regard to ADP-ribose removal? Similarly, would the biological modules that bind ADP-ribose groups, such as a macrodomain, have substrate or amino acid binding specificity? Notably, the specificities of macrodomains have been shown to be dependent on the amino acids surrounding the modified sites [66, 74]. Thus, these macrodomains will likely enrich for a restricted set of endogenous ADP-ribosylated proteins. Recently, by comparing the ADP-ribosylated proteome from human and mouse cells before and after enrichment by the Af1521 macrodomain, our group found that the macrodomain-enriched proteome selects against ADP-ribosylated glutamate residues globally [32], consistent with the earlier findings that this macrodomain bears hydrolase activity against acidic MARYlated amino acids of a single substrate [7, 75]. It can be postulated, then, that the glutamate sites identified following enrichment by Af1521 macrodomain were PARYlated prior to enrichment, as MAR would have been hydrolyzed off. Using this same line of reasoning, binding and hydrolase specificity (for both the targeted ADP-ribosylated residues and neighboring amino acids) of all ADP-ribose binding modules can be systematically defined. Table 4-1 summarizes the binding affinity and substrate specificity of

some of the most-studied ADP-ribose binding domains and hydrolases. While the primary aim of these characterization studies is often to elucidate the role these protein domains play in cell biology, they have also provided a much-needed expansion of a ‘biological toolbox’ for distinguishing between classes of ADP-ribosylated substrates, an effort which began 20 years ago with the ARH1-aided classification of substrates carrying MARYlation on arginine residues [76]. This toolbox should provide the means for enriching targeted groups of ADP-ribosylated proteins to expand our knowledge of the ADP-ribosylated proteome.

4.7 Distinguishing between sites of MAR- and PARylation

While it is advisable — and at this point only possible — to study the attachment sites of all forms of ADP-ribosylation together, the distinction between MAR and PAR, as well as the many subclasses of PAR, will likely prove critical for interpretation of the role played by the modified residue of interest. For example, five out of the 15 enzymatically active human PARPs are responsible for PARylation activity, with the other 10 restricted to MARYlation (Figure 4-1), meaning that a change in the PARylation status of a residue can only be attributed to the enzymatic activity of those five PARPs. A similar analysis could be employed for the ADP-ribosyl hydrolases: two can only remove MAR (macroD1 and macroD2), two can turn PAR into MAR (PARG and ARH3) and one can remove both PAR and MAR (TARG1; see table 4-1) [77]. Therefore, understanding how an ADP-ribosylation site is changing between an unmodified state and carrying MAR or PAR can suggest the enzymes responsible for its regulation. The clinical implications of understanding the distinction between PAR vs. MAR is exemplified in the PARP inhibitor classification performed by Wahlberg et al., where 185 PARP inhibitors were assayed for their abilities to bind members of the human PARP family; many of these inhibitors bind to MARYlating as well as PARylating members of the family [12]. Such potential off-target inhibition of MARYlation would not be revealed by the typical assay for monitoring the effectiveness of PARP inhibitors, which only measures changes in PARylation level. Knowing which ADP-ribosylation sites are affected by these inhibitors (or in disease states) and how those ADP-ribosylation sites are changing between unmodified, MARYlated

and PARylated will be predictive of the PARPs targeted in cells. Finally, multiple ADP-ribose groups in PAR may define functional roles distinct from MAR. For example, while wild-type, PARylation-capable PARP-1 is able to fully rescue DNA repair in PARP-1^{-/-} MEFs, a PARP-1 mutant that is only capable of MARYlation activity cannot [16]. Such detrimental changes brought on by converting PARylation to MARYlation sites may be because the structure of PAR is similar to that of polynucleic acids (e.g. DNA) and thus could compete for, or modulate the functions of, factors that bind nucleic acids. For these reasons, we will now examine potential methods for classifying sites of ADP-ribosylation based on the structure of their PTM.

As diagrammed in Figure 4-4B, MAR is a homogenous modification with a predictable mass of 541.06 Daltons, allowing MARYlation site localization by MS. Given that MARYlated peptides can be captured by phosphopeptide enrichment techniques [78], it is feasible to globally enrich MARYlated peptides from protease-digested cell lysates. In fact, re-analysis of phosphoproteomic data uncovered 79 MARYlated proteins [79]. However, this re-analysis likely underestimates the global level of MARYlation due to the high pH (pH 10) phosphopeptide elution employed [80], a condition which results in loss of ADP-ribose groups conjugated to acidic sites [15]. Consistently, all but one of the MARYlated sites identified in the re-analysis were arginine, an observation which was partly attributed to the increased stability of ADP-ribosylated arginine as opposed to ADP-ribosylated glutamate in the conditions employed for their study [79]. For non-biased detection of MARYlated proteomes, the labile bond between ADP-ribose groups and acidic amino acids must be preserved, e.g. by choosing a neutral phosphate buffer for eluting the phosphopeptide enrichment matrices (as in [32]).

Another possibility to distinguish MARYlated substrates from PARylated substrates is to exploit the distinct properties of protein domains that specifically recognize them (see biological toolbox, Table 4-1). For example, the WWE domain recognizes iso-ADP-ribose — the molecular structure spanning consecutive ADP-ribose subunits of PAR [81]; therefore this domain could be an

ideal tool for enriching PARylated, but not MARylated, targets. Alternatively, MacroD2 can be engineered to abrogate its inherent ADP-ribose hydrolase activity but retain its binding specificity towards MARylated substrates [75]. However, most of these domains were tested with single MARylated or PARylated substrates. Use of this biological toolbox for proteome-wide investigation warrants systematic analyses of these ADP-ribose binding modules to fully characterize their substrate specificities for both binding and hydrolysis.

4.8 Free/Conjugated, Branched/Linear: the many forms of Poly(ADP-ribose)

Besides identifying the ADP-ribosylation sites, MS can also be used to accurately quantitate PAR levels with femtomole sensitivity [82]. Assuming an average chain length of 10 ADP-ribose units per PAR molecule, the Bürkle group estimated that there are about 3,000 PAR molecules/cell in native cellular conditions, which can be induced to >150,000 molecules/cell upon DNA damage, with a branching frequency of 1–2% [82]. Combining this methodology with site identification could allow researchers to deduce whether the increase in PARylation is a result of new PARylation sites and/or substrates, or simply elongation of existing sites on existing substrates. However, one should be aware of an alternative source of PAR — the soluble PAR that is not attached to target proteins. The existence of soluble PAR *in vivo* has been inferred from mounting evidence that PARG has both endo- and exo-glycosidic activity, allowing this enzyme to produce and regulate levels of free PAR [77]. Additionally the ADP-ribosyl hydrolase TARG1 has been shown to reduce PARylation levels on auto-modified PARP-1 without releasing free ADP-ribose *in vitro* [83], indicating that the entire PAR chain could be released as a single unit in cells. The cellular implications of free PAR were demonstrated by the release of apoptosis-inducing factor (AIF) following exposure of cells to free PAR, an effect which was not observed in the presence of digested PAR [84]. Finally, the ADP-ribosyl hydrolase ARH3, which degrades PAR, regulates the release of AIF in cells, hypothetically through its ability to degrade free PAR [85]. Notably, cellular PAR levels are an important clinical parameter to measure the effectiveness of PARP inhibitors and/or chemotherapeutic agents in

clinical trials (as in NCI standard operating procedure #340505) as well as a predictive biomarker proposed for PARP inhibitor sensitivity [86, 87]. An understanding of the conjugation state of cellular PAR is necessary for accurate interpretation of changing PARylation levels.

Current approaches do not account for another important parameter — the structural subclasses of PAR. These subclasses include length variants [1, 2] as well as branching variants (PARPs 1 and 2 make branched polymers while 5a makes linear polymers [88-90]). These differences could functionally impact PAR's role as a scaffold, where different lengths of the polymer have already been shown to recruit distinct populations of proteins (e.g. [91]) — a potential mechanism for temporal coordination of cellular processes [92, 93]. The development of proteomic tools to determine polymer length and structure in cells could shed light on the unique roles played by the many forms of PAR. The recent development of a purification scheme for large amounts of PAR standards of defined length [37] could potentially pave the way for characterizing the length of the polymer on PARylated substrates. Ultimately, the goal is to use MS to simultaneously identify both the sites of ADP-ribosylation and the number of ADP-ribose groups that are attached to those modified sites. Such technical challenges bear remarkable similarity to the problem of the site-specific microheterogeneity observed in N-linked glycosylation, where structures of sugar polymer attached to the modified sites could be of different lengths and varied degrees of branching [94]. Recent advances in search algorithms have been able to map simultaneously the glycosylation sites, the number of sugar moieties and the branch points of the sugar polymer attached at the modified site of single proteins [95]. Though an ADP-ribose moiety carries more negative charge and generally two-fold more mass than sugar moieties, it is perhaps feasible to map both the modified sites and short oligomers (<15mers) attached on single PARylated proteins in the future.

4.9 Assessing the physiological relevance of ADP-ribosylation sites

4.9.1 Site Occupancy

Complete characterization of a single ADP-ribosylation site will include accurate identification of four factors: (1) amino acid conjugation site, (2) enzymes responsible for addition

and removal of the modification, (3) structural make-up of the modification (mono? poly? branched?), and (4) site occupancy/stoichiometry. While progress has been made in the first three endeavors as discussed above, it is the last aim which will most aid in the determination of functionally and physiologically relevant sites of ADP-ribosylation; functional (and therefore regulated) sites will likely exhibit a defined stoichiometric change in response to stimulus, while non-functional sites will show no change or changes that cannot be associated consistently with the biological stimulus applied. Quantifying a change in site occupancy, however, is much more challenging than quantifying a change in protein levels as the measurement may track the changing intensity of a single peptide as opposed to many peptides from a single protein [96]. Additionally many of the modifications may exist at very low stoichiometries, making quantification extra sensitive to variability introduced during sample preparation, a challenge which has been mitigated by the use of internal, stable-isotope labeled standards [97, 98]. Investigation of site occupancies (and the identification of robust, reproducible changes at determined sites) has the potential to test two hypotheses: (1) that some protein/peptide N-terminal, lysine, arginine, and cysteine modifications may be non-functional (and therefore represent biological noise), as they have the potential to be formed non-enzymatically by ADP-ribose groups that are released from PAR degradation by PARG/ARH3 and/or NADase activity of PARPs [44, 59, 60] and (2) that ADP-ribosylation of proteins is not always residue-specific, and may occasionally be mapped to a protein region as opposed to an amino acid. This latter hypothesis has been proposed to explain PARP-1 PARylation of BRCA1, wherein regions of BRCA1 were identified as PARylation acceptors as opposed to sites [99]. This observation stands in contrast to PARP-1 mediated PARylation of the tumor suppressor p53, of which mutational analysis has yielded three p53 PARylation sites that account for nearly all of the PARylation present on the substrate [100]. Mutating all three residues to alanine resulted in cytoplasmic accumulation of p53 and further biochemical experiments indicated that this site-specific PARylation on p53 blocked its interaction with the nuclear export receptor Crm1 [100]. Both region-specific as well as site-specific mechanisms appear to be at play following PARP-1 auto-modification,

an event which has been carefully characterized by a number of MS studies in recent years, resulting in a large number of site identifications (see Figure 4-6, source data in Supplementary Table 4-4) [31, 32, 55, 62, 83]. While several defined modification sites such as E488 and E491 have been identified by all studies there are also regions -- such as the C-terminus of the WGR domain stretching from E642-E650 -- which show regional, but not necessarily site-specific, overlap between studies. The ability to monitor whether sites or protein regions exhibit the regulatory patterns associated with cellular changes will provide essential data for determining their relative importance.

4.9.2 Top-down Proteomics

A necessary step forward will come from linking ADP-ribosylation into the established network of integrated PTMs [101]. Some work has already been done to link PARylation and ubiquitination [102], as well as ADP-ribosylation and acetylation [103], elucidating important cellular mechanisms. Future findings will be brought on by the constant development of MS analysis software, a critical component in PTM identification, as well as the increasing availability of liquid chromatography methods and mass analyzers that are compatible with top-down proteomics. As top-down proteomics analyzes intact proteins (rather than the peptides which result from proteolysis), this method can often distinguish between protein proteoforms, i.e. gene products that are post-translationally processed in multiple ways, often with functional implications [104, 105]. This technique has proven powerful in the analysis of complex proteoforms such as histone variants, enabling the simultaneous characterization of the 14 H2A proteoforms [106], and more recently, whole-protein kinetics of acetylation turnover on histones H3, H4 and H2A [107]. In the same way, top-down proteomics could facilitate the identification of groups of temporally or spatially correlated ADP-ribosylation sites, as well as other protein modifications. Integration of ADP-ribosylation into the growing network of PTMs has the potential to reveal novel regulatory roles for ADP-ribosylation and provide context for the physiological changes brought on by its modulation.

4.10 Conclusions

The power to monitor and interpret proteome-wide changes in ADP-ribosylation states promises to advance the fundamental understanding of ADP-ribosylation biology and facilitate further connections between cellular and patient responses to therapeutic PARP inhibition. The depth of the proteome will clearly be advanced with the invention of better tools to enrich ADP-ribosylated proteomes — MAR/PAR-binding proteomes, MAR/PARYlated proteomes and PARP-specific proteomes from cells in different cellular conditions, particularly native conditions which are understudied due to their low levels of ADP-ribosylation. However, such procurement of vast amounts of data must be coupled with the urgency to address basic questions such as whether the site of the PTM attachment matters, whether the PTM is always added enzymatically, and what the functional consequences are of adding single vs. multiple ADP-ribose residues onto the attachment site. In light of the promise shown by these new proteomic tools for the study of ADP-ribosylation, it is high time to investigate this therapeutically important, yet enigmatic, protein modification at a detailed mechanistic level.

4.11 Acknowledgements

We thank Drs. Phil Sharp, Paul Chang, Rhoel Dinglasan, Michael Cohen, Andrew Holland, Ted Dawson and Michael Matunis for critical reading of the manuscript. The proteomics work in the Leung and Ong Laboratories have been supported by a DOD Breast Cancer Research Program Idea Award #BC101881 (A.K.L.L.), an NIH grant R01-GM104135 (A.K.L.L.), the Safeway Research Foundation (A.K.L.L.), the Patrick C. Walsh Prostate Cancer Research Fund (A.K.L.L.), the Allegheny Health Network–Johns Hopkins Cancer Research Fund (A.K.L.L.), a Journal of Cell Science Travelling Fellowship (C.M.D), a Joy Cappel Young Investigator Award (C.M.D), an NCI training grant 5T32CA009110 (C.M.D), and an NIDA grant P30DA028846-01P30-DA028846 (S.-E.O).

4.12 Figures

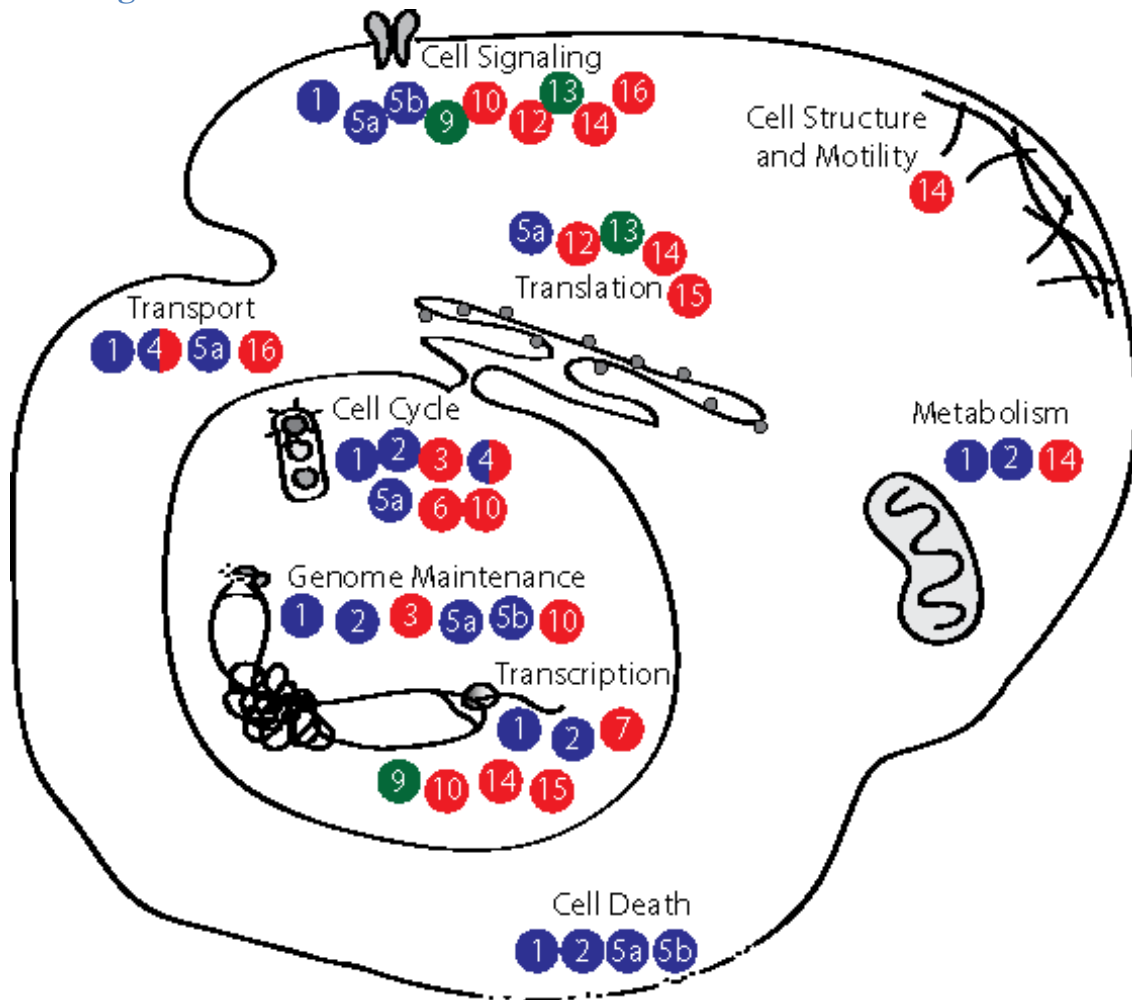


Figure 4-1 The PARP family

PARPs have been linked to nearly all major cellular processes. Juxtaposition of protein identifiers (e.g. 1 = PARP-1) indicates the involvement of the protein in the regulation or execution of the cellular process. Enzymatic activity is indicated by the bubble color: blue = poly(ADP-ribosyl)transferase, red = mono(ADP-ribosyl)transferase, green = no transferase activity. For references see Supp. Table 4-1.

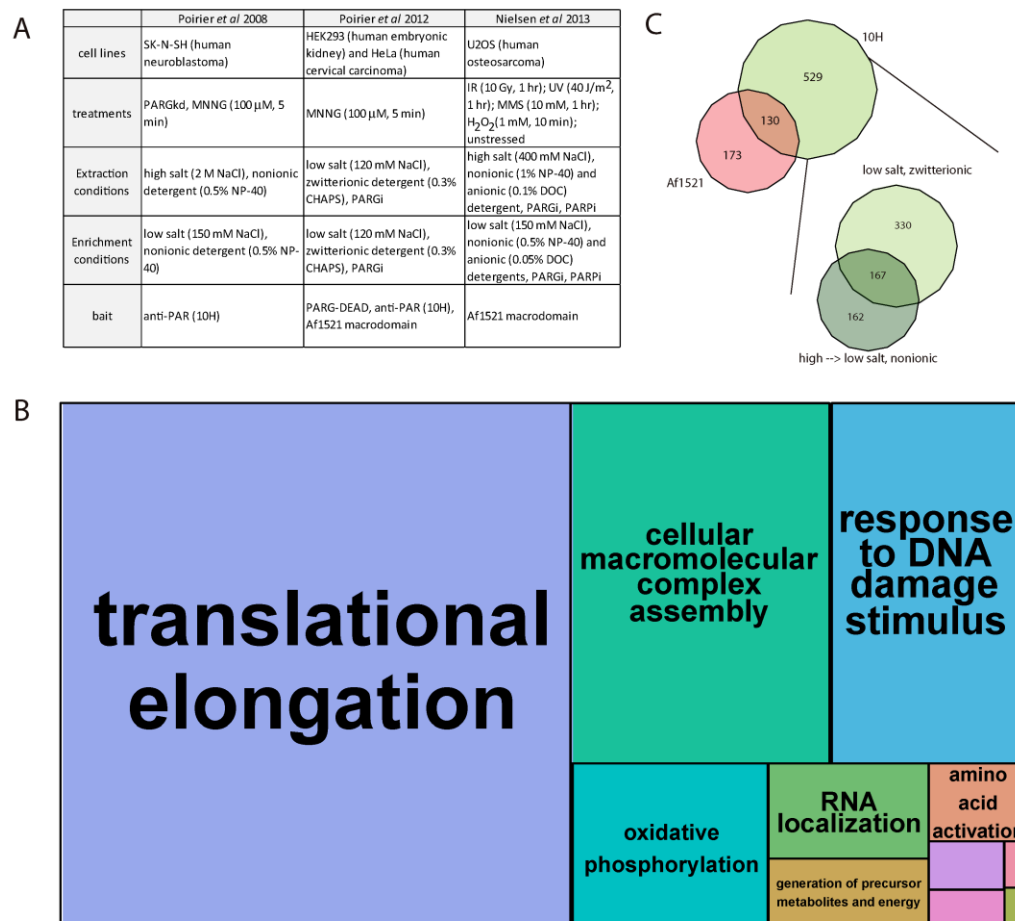


Figure 4-2 Processes enriched in the ADP-ribosylated interactome

(A) Experimental design for the interactome studies used for this meta-analysis. (B) A compilation of the proteins identified in response to DNA damage can be broken out by enrichment methods (bait) or cell lysis conditions. For comparison of lysis conditions the 10H enriched proteins were compared. Euler diagrams created in VennMaster [3, 108]. (C) The pooled DNA-damaged induced ADP-ribosylated interactome depicted as a treemap of enriched biological processes. The most enriched biological processes (based on statistical likelihood) are shown as larger components within the map, and grouped according to common cellular functions. Gene ontology determined using DAVID [109], treemap constructed using REVIGO [110] and R [111]. PARGi, PARG inhibitor; PARPi, PARP inhibitor. Data available in Supplementary Table 4-2.

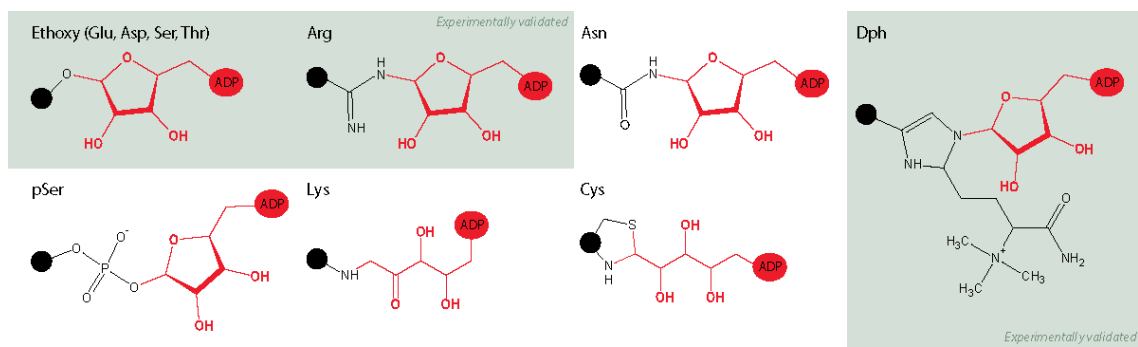


Figure 4-3 ADP-ribosylation attachment sites

Known and predicted structures linking amino acids to ADP-ribose, grayed out boxes show structures which have been validated. See text for references.

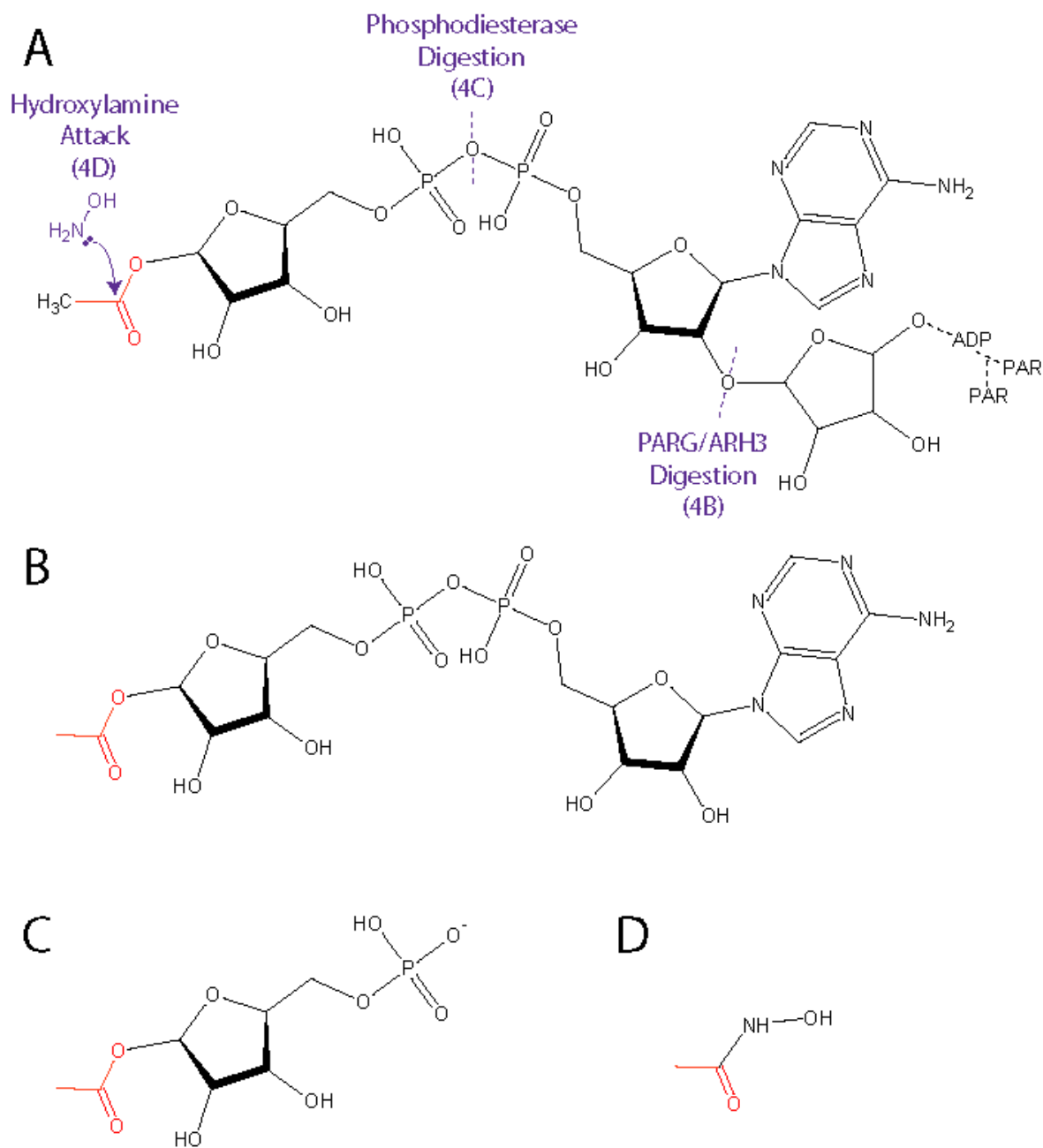


Figure 4-4 ADP-ribosylation tags

(A) Poly(ADP-ribose) can be simplified to mono(ADP-ribose) as in (B) by the glycohydrolase activity of PARG/ARH3, (C) to phosphoribose through digestion by phosphodiesterase, or (D) to a hydroxamic acid derivative though exposure to hydroxylamine. Of note, hydroxylamine treatment on ADP-ribosylated arginine results in the formation of the hydroxymate of ADP-ribose [50] and

therefore will likely not leave the 15.01 Da signature on formerly modified arginine residues as in glutamate/aspartate residues [31]. A representative acidic attachment site (red) is used for illustration.

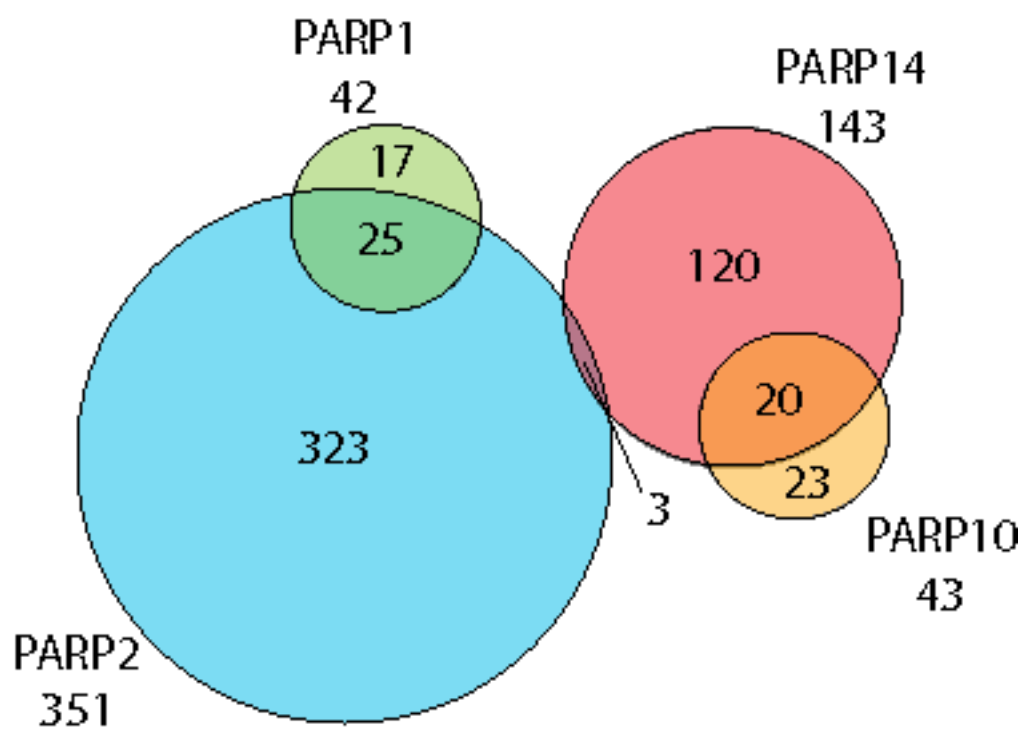


Figure 4-5 PARP Substrate Specificity

Substrates for PARP-1, PARP-2, PARP-10 and PARP-14 were identified in three studies using protein arrays or analogue sensitive mutant protein identification (see text). Euler diagrams created in VennMaster [3, 108]. Source data available in Supplementary Table 4-3.

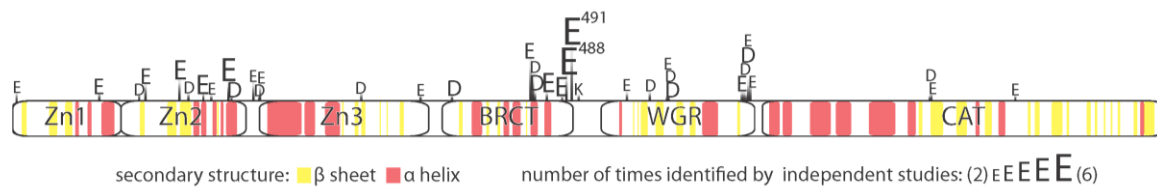


Figure 4-6 PARP-1 automodification sites

Schematic of PARP-1 includes protein domains and secondary structure; α helices are shown in red, β sheets in yellow. Auto-modification sites identified by at least two independent studies are shown. Size of annotated residues based on number of independent research groups which have identified the modification site. E488 and E491 located at the C-terminus of the BRCT domain are identified by all MS studies are shown as the two major auto-modification sites. For references see text and Supplementary Table 4-4.

Protein	Domain	binds		removes		mutant known		References
		MAR	PAR	MAR	PAR	binding	hydrolase	
macroH2A1.1	MD						N/A	1
ALC1	MD						N/A	2
SARS-CoV nsp3	MD						N/A	3
HEV ORF1	MD						N/A	4
Af1521	MD			E				5
macroD1	MD			E				6
macroD2	MD			E				7
PARP14	MD1						N/A	8
PARP14	MD2						N/A	9
various	PBZ						N/A	10
RNF146	WWE						N/A	11
SFV nsp3	MD						N/A	12
PARG	MD							13
C6orf130/TARG1	MD			E		N/A		14
ARH1	unknown			R		N/A		15
ARH3	unknown					N/A		16

Table 4-1 A biological toolbox of ADP-ribose binding and hydrolysis protein domains

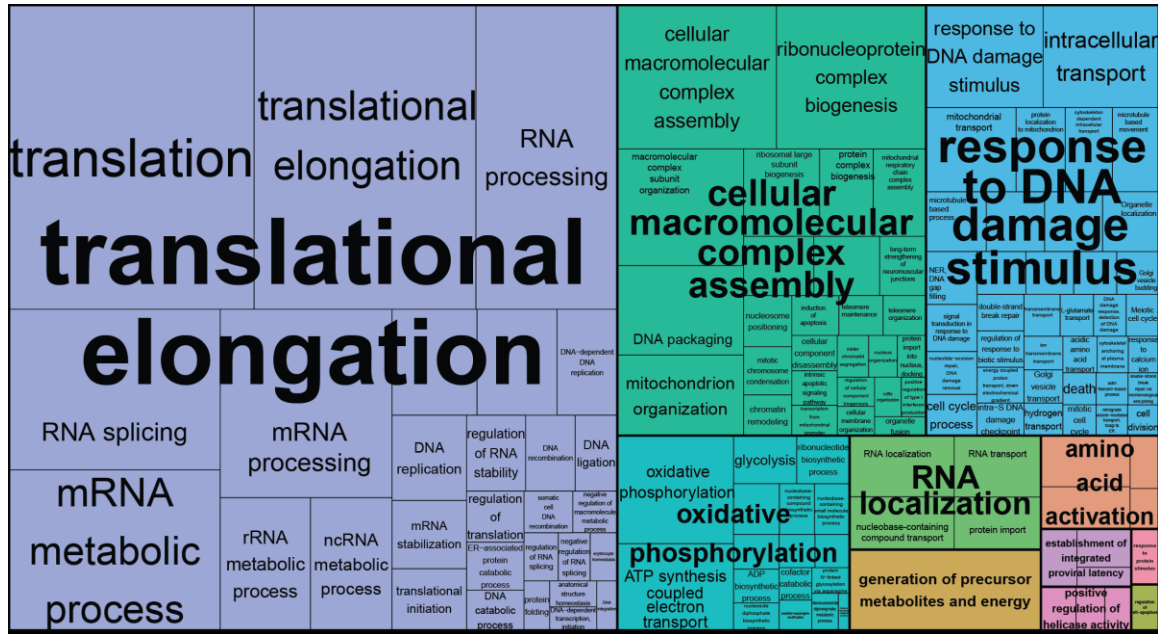
Our current understanding of the most well-studied ADP-ribose binding domains and hydrolases.

Green = Yes, Red = No, E/R = hydrolysis shown specifically for glutamic acid or arginine residues, respectively. MD = macrodomain, N/A = not applicable, blank = possible but currently unknown.

SARS-CoV, Severe Acute Respiratory Syndrome-Coronavirus; HEV, Hepatitis E Virus; SFV, Semliki Forest Virus.

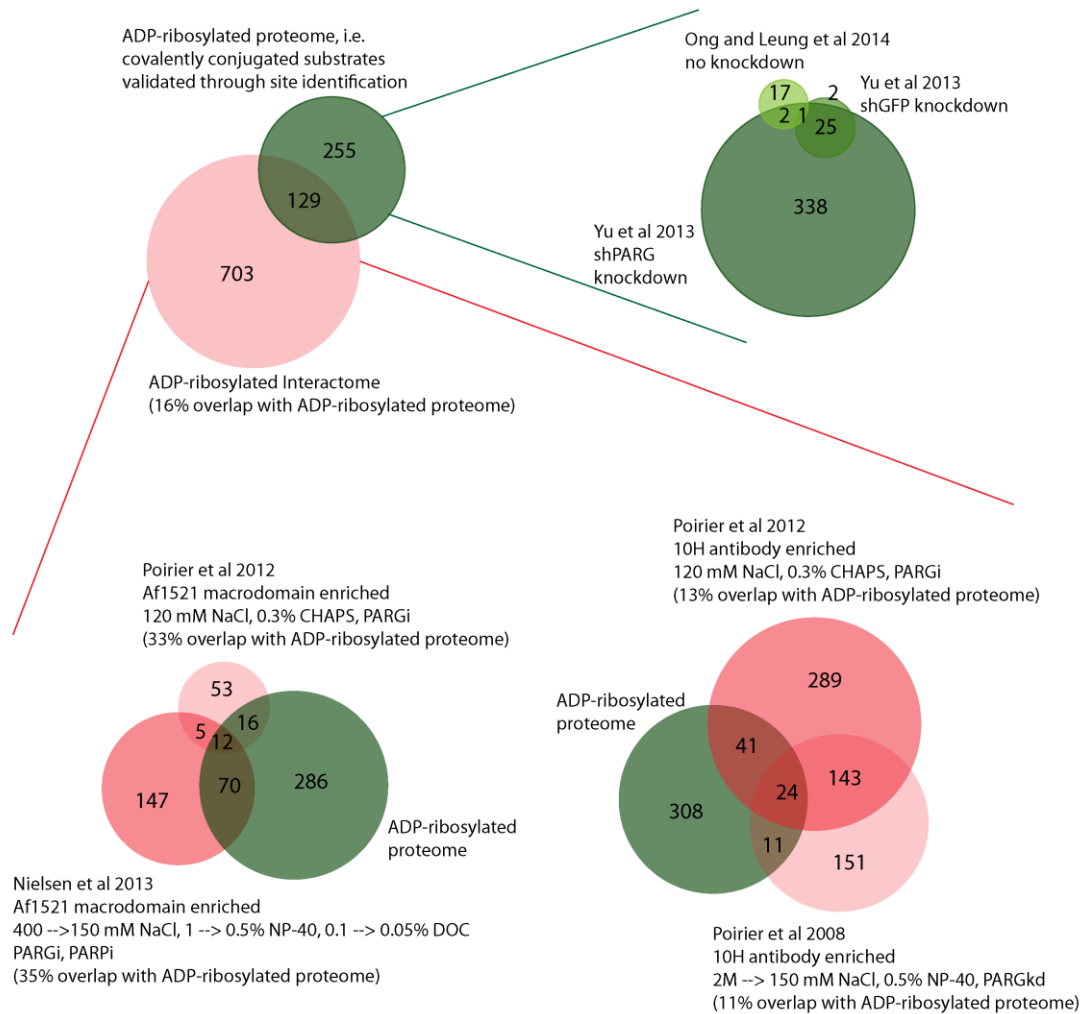
References:¹[7, 75, 112], ²[30, 113], ^{3&4}[114, 115], ⁵[7, 30, 32], ⁶[7, 75, 115], ⁷[7, 115], ^{8&9}[7, 74], ¹⁰[116-118], ¹¹[81], ¹²[114, 115], ¹³[7, 20, 38], ¹⁴[7, 83], ¹⁵[119, 120], ¹⁶[120]

4.13 Supplementary Figures



Supplementary Figure 4-1 Processes enriched in the ADP-ribosylated interactome (full table of Figure 2B).

Gene ontology determined using DAVID, treemap constructed using REViGO and R. Source data available in Table S2.



Supplementary Figure 4-2 Comparing the ADP-ribosylated interactomes with the known ADP-ribosylated proteomes.

PARGi, PARG inhibitor; PARPi, PARP inhibitor; PARGkd, PARG knockdown. Interactome studies, including protein extraction and enrichment conditions, are described in detail in Figure 2A. Source data available in Supplementary Table 4-2.

4.14 Supplementary Text

A cautionary note—the possibility of non-enzymatic ADP-ribosylation

Non-enzymatic modification of amino acids by ADP-ribose has been identified by several pioneers in the field, including studies by Drs. Elaine and Myron Jacobson (e.g. Cervantes-Laurean et al., 1996), Joel Moss (e.g. McDonald and Moss, 1994), and mentioned in reviews by Drs. Alexander Bürkle (Bürkle, 2005), Guy Poirier (D'Amours et al., 1999) and Michael Hottiger (Hassa et al., 2006). These original studies clearly indicated the possibility for ADP-ribose to non-enzymatically conjugate to lysine, arginine and cysteine residues *in vitro*, using different analytical tools, including NMR, chromatography profile, and sensitivities to different chemicals. As stated in Cervantes-Laurean et al., 1996, the chemical conjugation of ADP-ribose (also known as glycation) was added to proteins in a concentration-dependent manner and can occur even within 8 minutes (the shortest timepoint sampled). Figure 5 in this 1996 paper further illustrated different rates of chemical ADP-ribosylation for different histones that have different numbers of lysines (e.g. H1 with 56 lysines incorporated less than H4 with 11 lysines), suggesting that the incorporation may not be random. Drawing a parallel to non-enzymatic glycation by glucose, only specific lysine residues in albumin are chemically conjugated *in vivo* (Iberg and Flückiger, 1986), therefore it is not clear whether each lysine is equally non-enzymatically modified.

Given the highly sensitive nature of state-of-the-art mass spectrometry, the stable nature of ADP-ribose-lysine/arginine bond and the use of enrichment tools to identify ADP-ribosylated sites, it is experimentally feasible to detect even low level of artificial “noise” from biological samples prepared for proteomics studies. We would like to note that ADP-ribose can be generated by (1) PARG/ARH3 conversion of PARylation sites to MARYlation sites for site identification and/or potentially *in vivo* (Messner et al., 2010; Rosenthal et al., 2011), or (2) PARPs when acting as NADases (e.g., Desmarais et al., 1991). In addition, ADP-ribose has been used as an elution condition for macrodomain enrichment for ADP-ribosylated proteins (Dani et al., 2009), which would not be a problem in identifying substrates; however, the excessive amount of free ADP-ribose

used for elution could potentially chemically conjugate the substrates in the eluates *in vitro*, resulting in the identification of non-physiological ADP-ribosylation sites by mass spectrometry. Given that the effect of chemical conjugation by ADP-ribose in proteomic studies remains uncertain, the field would greatly benefit from performing a series of experiments to clearly establish whether or not non-enzymatic ADP-ribosylation should be a concern for proteomics studies.

Criteria for proteomics datasets analyzed in Supplementary Table 2

We have analyzed all four proteomic studies undertaken so far that identify ADP-ribosylated substrates along with their interactors (i.e., the ADP-ribosylated interactome) (Gagné et al., 2008; 2012; Isabelle et al., 2012; Jungmichel et al., 2013). We note that the Jungmichel et al 2013 study is aimed at mapping ADP-ribosylated targets (substrates) during DNA damage. However, based on the materials and methods in Jungmichel et al (2013), PAR enrichment was performed in cell lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.05% sodium deoxycholate, protease inhibitors, phosphatase inhibitors, PARG inhibitor and PARP inhibitor. This buffer most closely resembles the modified RIPA buffer commonly used in proteomics for protein–protein interactome enrichment (e.g. by the Matthias’ Mann group in Ong and Mann, 2006), though contains less of the ionic detergent which represents the harshest denaturant in the buffer (0.05% sodium deoxycholate vs. 0.25% in Ong and Mann, 2006). Standard RIPA buffer, which contains 1% sodium deoxycholate, is also commonly employed for the study of protein-protein interactions (Sefton, 2001). Therefore, conservatively, it can reasonably be assumed that the proteins identified in this study constitute ADP-ribosylated interactome, i.e. ADP-ribosylated proteins, ADP-ribose interactors, and protein–protein interaction partners of both the former and latter. However, we noted that the lysis conditions used are slightly more stringent than those used by Poirier and co-workers in their 2008 and 2012 studies as summarized in Figure 2A (Gagné et al., 2008; 2012; Isabelle et al., 2012). Using the current set of 384 ADP-ribosylated substrates with defined sites mapped (Daniels et al., 2014; Zhang et al., 2013), 82 of these proteins overlap with the Jungmichel 2013 data,

accounting for ~35% of their Af1521 macrodomain-enriched proteome. On the other hand, there are 28 proteins overlapping with the Gagné 2012 data, accounting for a comparable ~33% of their Af1521 macrodomain-enriched proteome (see Supplementary Figure 2 below). The differences in induction, lysis and enrichment conditions, cell lines as well as limited sampling, in general, could all contribute to the different degrees of overlap. The actual proportion of ADP-ribosylated substrates vs. binding proteins found in these respective studies can be determined with approaches that identify specific sites of ADP-ribosylation in future studies. Given that the condition reported in Jungmichel et al (2013) cannot exclude the inclusion of proteins that binds to ADP-ribosylated substrates, we therefore take a conservative approach and discuss the paper in the context among other pioneering works of ADP-ribosylated interactomes.

In order to control the many variables present in the meta-analysis presented in Figure 2B, the proteins identified through PARG-dead enrichment were omitted, as they represent a single dataset, whereas the 10H (Gagné et al., 2008; 2012; Isabelle et al., 2012) and macrodomain (Gagné et al., 2008; Jungmichel et al., 2013) enriched protein groups represent at least two independent datasets.

4.15 Supplementary Tables

Table S1. Functions attributed to PARP family members.

Supporting examples from the literature for the connection made between each PARP and each cellular process in Figure 1.

Table S2. The ADP-ribose interactome

Sourced data used to generate the ADP-ribosylated interactomes shown in Figure 2. See Supplementary Text for criteria of data inclusion.

Table S3. PARP specific substrates

Sourced data used to generate the Euler diagrams showing PARP-1, PARP-2, PARP-10 and PARP-14 specific substrates in Figure 5.

Table S4. Auto-modification sites identified on PARP-1

Sourced data used to generate the PARP-1 auto-modification site map from Figure 6.

All supplemental tables available online:

<http://www.sciencedirect.com/science/article/pii/S1097276515004517>

4.16 References

1. Vyas, S., et al., *Family-wide analysis of poly(ADP-ribose) polymerase activity*. Nat Commun, 2014. **5**: p. 4426.
2. Hottiger, M.O., et al., *Toward a unified nomenclature for mammalian ADP-ribosyltransferases*. Trends Biochem Sci, 2010. **35**(4): p. 208-19.
3. Aredia, F. and A.I. Scovassi, *Poly(ADP-ribose): A signaling molecule in different paradigms of cell death*. Biochem Pharmacol, 2014.
4. Perraud, A.L., et al., *ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology*. Nature, 2001. **411**(6837): p. 595-9.
5. Feijs, K.L., P. Verheugd, and B. Luscher, *Expanding functions of intracellular resident mono-ADP-ribosylation in cell physiology*. FEBS J, 2013. **280**(15): p. 3519-29.
6. Gibson, B.A. and W.L. Kraus, *New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs*. Nat Rev Mol Cell Biol, 2012. **13**(7): p. 411-24.
7. Rosenthal, F., et al., *Macrodomain-containing proteins are new mono-ADP-ribosylhydrolases*. Nat Struct Mol Biol, 2013. **20**(4): p. 502-7.
8. Rouleau, M., et al., *PARP inhibition: PARP1 and beyond*. Nat Rev Cancer, 2010. **10**(4): p. 293-301.
9. Steffen, J.D., et al., *Structural Implications for Selective Targeting of PARPs*. Front Oncol, 2013. **3**: p. 301.
10. Garber, K., *PARP inhibitors bounce back*. Nat Rev Drug Discov, 2013. **12**(10): p. 725-7.
11. Lord, C.J., A.N. Tutt, and A. Ashworth, *Synthetic Lethality and Cancer Therapy: Lessons Learned from the Development of PARP Inhibitors*. Annu Rev Med, 2014.
12. Wahlberg, E., et al., *Family-wide chemical profiling and structural analysis of PARP and tankyrase inhibitors*. Nat Biotechnol, 2012. **30**(3): p. 283-8.
13. Vyas, S., et al., *A systematic analysis of the PARP protein family identifies new functions critical for cell physiology*. Nat Commun, 2013. **4**: p. 2240.
14. Cox, J. and M. Mann, *Quantitative, high-resolution proteomics for data-driven systems biology*. Annu Rev Biochem, 2011. **80**: p. 273-99.
15. Cervantes-Laurean, D., E.L. Jacobson, and M.K. Jacobson, *Preparation of low molecular weight model conjugates for ADP-ribose linkages to protein*. Methods Enzymol, 1997. **280**: p. 275-87.
16. Mortusewicz, O., et al., *Feedback-regulated poly(ADP-ribosyl)ation by PARP-1 is required for rapid response to DNA damage in living cells*. Nucleic Acids Res, 2007. **35**(22): p. 7665-75.

17. Wielckens, K., et al., *DNA fragmentation and NAD depletion. Their relation to the turnover of endogenous mono(ADP-ribosyl) and poly(ADP-ribosyl) proteins*. J Biol Chem, 1982. **257**(21): p. 12872-7.
18. Hassa, P.O., et al., *Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going?* Microbiol Mol Biol Rev, 2006. **70**(3): p. 789-829.
19. Gagne, J.P., et al., *Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes*. Nucleic Acids Res, 2008. **36**(22): p. 6959-76.
20. Gagne, J.P., et al., *Quantitative proteomics profiling of the poly(ADP-ribose)-related response to genotoxic stress*. Nucleic Acids Res, 2012. **40**(16): p. 7788-805.
21. Jungmichel, S., et al., *Proteome-wide Identification of Poly(ADP-Ribosylation) Targets in Different Genotoxic Stress Responses*. Mol Cell, 2013.
22. Isabelle, M., et al., *Quantitative proteomics and dynamic imaging reveal that G3BP-mediated stress granule assembly is poly(ADP-ribose)-dependent following exposure to MNNG-induced DNA alkylation*. J Cell Sci, 2012. **125**(Pt 19): p. 4555-66.
23. Murawska, M., et al., *Stress-induced PARP activation mediates recruitment of Drosophila Mi-2 to promote heat shock gene expression*. PLoS Genet, 2011. **7**(7): p. e1002206.
24. Chang, P., M.K. Jacobson, and T.J. Mitchison, *Poly(ADP-ribose) is required for spindle assembly and structure*. Nature, 2004. **432**(7017): p. 645-9.
25. Boamah, E.K., et al., *Poly(ADP-Ribose) polymerase 1 (PARP-1) regulates ribosomal biogenesis in Drosophila nucleoli*. PLoS Genet, 2012. **8**(1): p. e1002442.
26. Leung, A.K., et al., *Poly(ADP-ribose) regulates stress responses and microRNA activity in the cytoplasm*. Mol Cell, 2011. **42**(4): p. 489-99.
27. Okano, S., et al., *Spatial and temporal cellular responses to single-strand breaks in human cells*. Mol Cell Biol, 2003. **23**(11): p. 3974-81.
28. Cardenas-Corona, M.E., E.L. Jacobson, and M.K. Jacobson, *Endogenous polymers of ADP-ribose are associated with the nuclear matrix*. J Biol Chem, 1987. **262**(31): p. 14863-6.
29. Kawamitsu, H., et al., *Monoclonal antibodies to poly(adenosine diphosphate ribose) recognize different structures*. Biochemistry, 1984. **23**(16): p. 3771-7.
30. Karras, G.I., et al., *The macro domain is an ADP-ribose binding module*. EMBO J, 2005. **24**(11): p. 1911-20.
31. Zhang, Y., et al., *Site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome*. Nat Methods, 2013. **10**(10): p. 981-4.

32. Daniels, C.M., S.E. Ong, and A.K. Leung, *A phosphoproteomic approach to characterize protein mono and poly(ADP-ribosyl)ation sites from whole cell lysate*. J Proteome Res, 2014.
33. Cortes, U., et al., *Depletion of the 110-kilodalton isoform of poly(ADP-ribose) glycohydrolase increases sensitivity to genotoxic and endotoxic stress in mice*. Mol Cell Biol, 2004. **24**(16): p. 7163-78.
34. Min, W., et al., *Deletion of the nuclear isoform of poly(ADP-ribose) glycohydrolase (PARG) reveals its function in DNA repair, genomic stability and tumorigenesis*. Carcinogenesis, 2010. **31**(12): p. 2058-65.
35. Finch, K.E., et al., *Selective small molecule inhibition of poly(ADP-ribose) glycohydrolase (PARG)*. ACS Chem Biol, 2012. **7**(3): p. 563-70.
36. Vivello, C.A. and A.K. Leung, *Proteomics approaches to identify mono(ADP-ribosyl)ated and poly(ADP-ribosyl)ated proteins*. Proteomics, 2014.
37. Tan, E.S., K.A. Krukenberg, and T.J. Mitchison, *Large-scale preparation and characterization of poly(ADP-ribose) and defined length polymers*. Anal Biochem, 2012. **428**(2): p. 126-36.
38. Slade, D., et al., *The structure and catalytic mechanism of a poly(ADP-ribose) glycohydrolase*. Nature, 2011. **477**(7366): p. 616-20.
39. Mao, Z., et al., *SIRT6 promotes DNA repair under stress by activating PARP1*. Science, 2011. **332**(6036): p. 1443-6.
40. Panzeter, P.L., B. Zweifel, and F.R. Althaus, *Synthesis of poly(ADP-ribose)-agarose beads: an affinity resin for studying (ADP-ribose)_n-protein interactions*. Anal Biochem, 1992. **207**(1): p. 157-62.
41. Kickhoefer, V.A., et al., *The 193-kD vault protein, VPARP, is a novel poly(ADP-ribose) polymerase*. J Cell Biol, 1999. **146**(5): p. 917-28.
42. Cervantes-Laurean, D., et al., *Protein modification by ADP-ribose via acid-labile linkages*. J Biol Chem, 1995. **270**(14): p. 7929-36.
43. Smith, J.A. and L.A. Stocken, *Chemical and metabolic properties of adenosine diphosphate ribose derivatives of nuclear proteins*. Biochem J, 1975. **147**(3): p. 523-9.
44. McDonald, L.J. and J. Moss, *Enzymatic and nonenzymatic ADP-ribosylation of cysteine*. Mol Cell Biochem, 1994. **138**(1-2): p. 221-6.
45. Manning, D.R., et al., *ADP-ribosylation of transducin by islet-activation protein. Identification of asparagine as the site of ADP-ribosylation*. J Biol Chem, 1984. **259**(2): p. 749-56.
46. Tsurumura, T., et al., *Arginine ADP-ribosylation mechanism based on structural snapshots of iota-toxin and actin complex*. Proc Natl Acad Sci U S A, 2013. **110**(11): p. 4267-72.
47. Altmeyer, M., et al., *Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites*. Nucleic Acids Res, 2009. **37**(11): p. 3723-38.

48. Oppenheimer, N.J. and J.W. Bodley, *Diphtheria toxin. Site and configuration of ADP-ribosylation of diphthamide in elongation factor 2*. J Biol Chem, 1981. **256**(16): p. 8579-81.
49. Moss, J., et al., *Molecular and immunological characterization of ADP-ribosylarginine hydrolases*. J Biol Chem, 1992. **267**(15): p. 10481-8.
50. Moss, J., D.A. Yost, and S.J. Stanley, *Amino acid-specific ADP-ribosylation*. J Biol Chem, 1983. **258**(10): p. 6466-70.
51. Adamietz, P. and H. Hilz, *Poly(adenosine diphosphate ribose) is covalently linked to nuclear proteins by two types of bonds*. Hoppe Seylers Z Physiol Chem, 1976. **357**(4): p. 527-34.
52. Messner, S. and M.O. Hottiger, *Histone ADP-ribosylation in DNA repair, replication and transcription*. Trends Cell Biol, 2011. **21**(9): p. 534-42.
53. Olsen, J.V., et al., *Global, in vivo, and site-specific phosphorylation dynamics in signaling networks*. Cell, 2006. **127**(3): p. 635-48.
54. Oetjen, J., S. Rexroth, and B. Reinhold-Hurek, *Mass spectrometric characterization of the covalent modification of the nitrogenase Fe-protein in Azoarcus sp. BH72*. FEBS J, 2009. **276**(13): p. 3618-27.
55. Tao, Z., P. Gao, and H.W. Liu, *Identification of the ADP-ribosylation sites in the PARP-1 automodification domain: analysis and implications*. J Am Chem Soc, 2009. **131**(40): p. 14258-60.
56. Hengel, S.M., et al., *Tandem mass spectrometry investigation of ADP-ribosylated kemptide*. J Am Soc Mass Spectrom, 2009. **20**(3): p. 477-83.
57. Messner, S., et al., *PARP1 ADP-ribosylates lysine residues of the core histone tails*. Nucleic Acids Res, 2010. **38**(19): p. 6350-62.
58. Rosenthal, F., et al., *Identification of distinct amino acids as ADP-ribose acceptor sites by mass spectrometry*. Methods Mol Biol, 2011. **780**: p. 57-66.
59. Kharadia, S.V. and D.J. Graves, *Relationship of phosphorylation and ADP-ribosylation using a synthetic peptide as a model substrate*. J Biol Chem, 1987. **262**(36): p. 17379-83.
60. Cervantes-Laurean, D., E.L. Jacobson, and M.K. Jacobson, *Glycation and glycoxidation of histones by ADP-ribose*. J Biol Chem, 1996. **271**(18): p. 10461-9.
61. Hengel, S.M. and D.R. Goodlett, *A Review of Tandem Mass Spectrometry Characterization of Adenosine Diphosphate-Ribosylated Peptides*. Int J Mass Spectrom, 2012. **312**: p. 114-121.
62. Chapman, J.D., et al., *Mapping PARP-1 auto-ADP-ribosylation sites by liquid chromatography-tandem mass spectrometry*. J Proteome Res, 2013.
63. Matsubara, H., et al., *Studies on poly (adenosine diphosphate ribose). V. Mechanism of hydrolysis of poly (adenosine diphosphate ribose) by snake venom phosphodiesterase*. J Biol Chem, 1970. **245**(14): p. 3606-11.

64. Oka, J., K. Ueda, and O. Hayaishi, *Snake venom phosphodiesterase: simple purification with Blue Sepharose and its application to poly(ADP-ribose) study*. Biochem Biophys Res Commun, 1978. **80**(4): p. 841-8.
65. Kistemaker, H.A., et al., *Stereoselective ribosylation of amino acids*. Org Lett, 2013. **15**(9): p. 2306-9.
66. Moyle, P.M. and T.W. Muir, *Method for the synthesis of mono-ADP-ribose conjugated peptides*. J Am Chem Soc, 2010. **132**(45): p. 15878-80.
67. van der Heden van Noort, G.J., et al., *Synthesis of mono-ADP-ribosylated oligopeptides using ribosylated amino acid building blocks*. J Am Chem Soc, 2010. **132**(14): p. 5236-40.
68. Szewczuk, L.M., M.K. Tarrant, and P.A. Cole, *Protein phosphorylation by semisynthesis: from paper to practice*. Methods Enzymol, 2009. **462**: p. 1-24.
69. Feijs, K.L., et al., *ARTD10 substrate identification on protein microarrays: regulation of GSK3beta by mono-ADP-ribosylation*. Cell Commun Signal, 2013. **11**(1): p. 5.
70. Troiani, S., et al., *Identification of candidate substrates for poly(ADP-ribose) polymerase-2 (PARP2) in the absence of DNA damage using high-density protein microarrays*. FEBS J, 2011. **278**(19): p. 3676-87.
71. Carter-O'Connell, I., et al., *Engineering the substrate specificity of ADP-ribosyltransferases for identifying direct protein targets*. J Am Chem Soc, 2014. **136**(14): p. 5201-4.
72. Guettler, S., et al., *Structural basis and sequence rules for substrate recognition by Tankyrase explain the basis for cherubism disease*. Cell, 2011. **147**(6): p. 1340-54.
73. Desmarais, Y., et al., *Enzymological properties of poly(ADP-ribose)polymerase: characterization of automodification sites and NADase activity*. Biochim Biophys Acta, 1991. **1078**(2): p. 179-86.
74. Forst, A.H., et al., *Recognition of mono-ADP-ribosylated ARTD10 substrates by ARTD8 macrodomains*. Structure, 2013. **21**(3): p. 462-75.
75. Jankevicius, G., et al., *A family of macrodomain proteins reverses cellular mono-ADP-ribosylation*. Nat Struct Mol Biol, 2013. **20**(4): p. 508-14.
76. Ohno, T., et al., *Detection of arginine-ADP-ribosylated protein using recombinant ADP-ribosylarginine hydrolase*. Anal Biochem, 1995. **231**(1): p. 115-22.
77. Barkauskaite, E., et al., *The recognition and removal of cellular poly(ADP-ribose) signals*. FEBS J, 2013. **280**(15): p. 3491-507.
78. Laing, S., et al., *Strategies for the identification of arginine ADP-ribosylation sites*. J Proteomics, 2011. **75**(1): p. 169-76.
79. Matic, I., I. Ahel, and R.T. Hay, *Reanalysis of phosphoproteomics data uncovers ADP-ribosylation sites*. Nat Methods, 2012. **9**(8): p. 771-2.

80. Huttlin, E.L., et al., *A tissue-specific atlas of mouse protein phosphorylation and expression*. Cell, 2010. **143**(7): p. 1174-89.
81. Wang, Z., et al., *Recognition of the iso-ADP-ribose moiety in poly(ADP-ribose) by WWE domains suggests a general mechanism for poly(ADP-ribosyl)ation-dependent ubiquitination*. Genes Dev, 2012. **26**(3): p. 235-40.
82. Martello, R., et al., *Quantification of cellular poly(ADP-ribosyl)ation by stable isotope dilution mass spectrometry reveals tissue- and drug-dependent stress response dynamics*. ACS Chem Biol, 2013. **8**(7): p. 1567-75.
83. Sharifi, R., et al., *Deficiency of terminal ADP-ribose protein glycohydrolase TARG1/C6orf130 in neurodegenerative disease*. EMBO J, 2013. **32**(9): p. 1225-37.
84. Yu, S.W., et al., *Apoptosis-inducing factor mediates poly(ADP-ribose) (PAR) polymer-induced cell death*. Proc Natl Acad Sci U S A, 2006. **103**(48): p. 18314-9.
85. Mashimo, M., J. Kato, and J. Moss, *ADP-ribosyl-acceptor hydrolase 3 regulates poly (ADP-ribose) degradation and cell death during oxidative stress*. Proc Natl Acad Sci U S A, 2013. **110**(47): p. 18964-9.
86. Gottipati, P., et al., *Poly(ADP-ribose) polymerase is hyperactivated in homologous recombination-defective cells*. Cancer Res, 2010. **70**(13): p. 5389-98.
87. Oplustilova, L., et al., *Evaluation of candidate biomarkers to predict cancer cell sensitivity or resistance to PARP-1 inhibitor treatment*. Cell Cycle, 2012. **11**(20): p. 3837-50.
88. Ame, J.C., et al., *PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase*. J Biol Chem, 1999. **274**(25): p. 17860-8.
89. Alvarez-Gonzalez, R. and M.K. Jacobson, *Characterization of polymers of adenosine diphosphate ribose generated in vitro and in vivo*. Biochemistry, 1987. **26**(11): p. 3218-24.
90. Rippmann, J.F., K. Damm, and A. Schnapp, *Functional characterization of the poly(ADP-ribose) polymerase activity of tankyrase 1, a potential regulator of telomere length*. J Mol Biol, 2002. **323**(2): p. 217-24.
91. Fahrner, J., et al., *Quantitative analysis of the binding affinity of poly(ADP-ribose) to specific binding proteins as a function of chain length*. Nucleic Acids Res, 2007. **35**(21): p. e143.
92. Aubin, R.J., et al., *Correlation between endogenous nucleosomal hyper(ADP-ribosyl)ation of histone H1 and the induction of chromatin relaxation*. EMBO J, 1983. **2**(10): p. 1685-93.
93. Leung, A.K., *Poly(ADP-ribose): an organizer of cellular architecture*. J Cell Biol, 2014. **205**(5): p. 613-9.

94. An, H.J., J.W. Froehlich, and C.B. Lebrilla, *Determination of glycosylation sites and site-specific heterogeneity in glycoproteins*. Curr Opin Chem Biol, 2009. **13**(4): p. 421-6.
95. Chandler, K.B., et al., *Exploring site-specific N-glycosylation microheterogeneity of haptoglobin using glycopeptide CID tandem mass spectra and glycan database search*. J Proteome Res, 2013. **12**(8): p. 3652-66.
96. Wu, R., et al., *A large-scale method to measure absolute protein phosphorylation stoichiometries*. Nat Methods, 2011. **8**(8): p. 677-83.
97. Kettenbach, A.N., J. Rush, and S.A. Gerber, *Absolute quantification of protein and post-translational modification abundance with stable isotope-labeled synthetic peptides*. Nat Protoc, 2011. **6**(2): p. 175-86.
98. Olsen, J.V., et al., *Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis*. Sci Signal, 2010. **3**(104): p. ra3.
99. Hu, Y., et al., *PARP1-driven poly-ADP-ribosylation regulates BRCA1 function in homologous recombination-mediated DNA repair*. Cancer Discov, 2014. **4**(12): p. 1430-47.
100. Kanai, M., et al., *Inhibition of Crm1-p53 interaction and nuclear export of p53 by poly(ADP-ribosyl)ation*. Nat Cell Biol, 2007. **9**(10): p. 1175-83.
101. Woodsmith, J., A. Kamburov, and U. Stelzl, *Dual coordination of post translational modifications in human protein networks*. PLoS Comput Biol, 2013. **9**(3): p. e1002933.
102. Kang, H.C., et al., *Iduna is a poly(ADP-ribose) (PAR)-dependent E3 ubiquitin ligase that regulates DNA damage*. Proc Natl Acad Sci U S A, 2011. **108**(34): p. 14103-8.
103. Liszt, G., et al., *Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase*. J Biol Chem, 2005. **280**(22): p. 21313-20.
104. Tran, J.C., et al., *Mapping intact protein isoforms in discovery mode using top-down proteomics*. Nature, 2011. **480**(7376): p. 254-8.
105. Smith, L.M., et al., *Proteoform: a single term describing protein complexity*. Nat Methods, 2013. **10**(3): p. 186-7.
106. Boyne, M.T., 2nd, et al., *Precise characterization of human histones in the H2A gene family by top down mass spectrometry*. J Proteome Res, 2006. **5**(2): p. 248-53.
107. Zheng, Y., P.M. Thomas, and N.L. Kelleher, *Measurement of acetylation turnover at distinct lysines in human histones identifies long-lived acetylation sites*. Nat Commun, 2013. **4**: p. 2203.
108. Kestler, H.A., et al., *Generalized Venn diagrams: a new method of visualizing complex genetic set relations*. Bioinformatics, 2005. **21**(8): p. 1592-5.

109. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists*. Nucleic Acids Res, 2009. **37**(1): p. 1-13.
110. Supek, F., et al., *REVIGO summarizes and visualizes long lists of gene ontology terms*. PLoS One, 2011. **6**(7): p. e21800.
111. Team, R.D.C., *R: A Language and Environment for Statistical Computing*. 2011.
112. Timinszky, G., et al., *A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation*. Nat Struct Mol Biol, 2009. **16**(9): p. 923-9.
113. Gottschalk, A.J., et al., *Activation of the SNF2 family ATPase ALC1 by poly(ADP-ribose) in a stable ALC1.PARP1.nucleosome intermediate*. J Biol Chem, 2012. **287**(52): p. 43527-32.
114. Egloff, M.P., et al., *Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains*. J Virol, 2006. **80**(17): p. 8493-502.
115. Neuvonen, M. and T. Ahola, *Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites*. J Mol Biol, 2009. **385**(1): p. 212-25.
116. Pleschke, J.M., et al., *Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins*. J Biol Chem, 2000. **275**(52): p. 40974-80.
117. Ahel, I., et al., *Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins*. Nature, 2008. **451**(7174): p. 81-5.
118. Oberoi, J., et al., *Structural basis of poly(ADP-ribose) recognition by the multizinc binding domain of checkpoint with forkhead-associated and RING Domains (CHFR)*. J Biol Chem, 2010. **285**(50): p. 39348-58.
119. Konczalik, P. and J. Moss, *Identification of critical, conserved vicinal aspartate residues in mammalian and bacterial ADP-ribosylarginine hydrolases*. J Biol Chem, 1999. **274**(24): p. 16736-40.
120. Oka, S., J. Kato, and J. Moss, *Identification and characterization of a mammalian 39-kDa poly(ADP-ribose) glycohydrolase*. J Biol Chem, 2006. **281**(2): p. 705-13.

5 Conclusions and Future Directions

Our work has produced an MS based pipeline for identifying ADP-ribosylation sites on proteins, as outlined in Figure 5-1. We have demonstrated the potential for this method through the identification of modification sites on endogenously MARYlated and PARylated proteins (see Chapter 2) at both acidic and basic amino acid residues, suggesting an unbiased means of detection. The prospects for future development and application of this pipeline are presented in this chapter.

5.1 Future Development - Exploring the ADP-ribosylated proteome to a greater depth

Our survey of the endogenous ADP-ribosylated proteome (see Chapter 2) identified 63 ADP-ribosylated residues from 21 human proteins and 33 murine proteins. A similar study using an alternative technique identified 1,048 acidic ADP-ribosylation sites on 340 human proteins [1], as this method is blind to all non-acidic protein conjugation events, and we uncovered a roughly equal number of acidic (31) and basic (32) site identifications, it can be reasonably assumed that the depth of the entire ADP-ribosylated proteome is substantially more than either study reported. In an effort to make our own protocol more robust, and thus capable of identifying more ADP-ribosylation sites from the same protein mixture, we have searched for an alternative enzyme for hydrolyzing protein-conjugated ADP-ribose to phosphoribose. Snake Venom Phosphodiesterase (SVP) – the pyrophosphatase used by our group – must be purified from snake venom (a recombinant purification scheme has not been reported) resulting in a multi-step purification process that is both cumbersome and produces lot-to-lot variability in the final product (see Chapter 3). Nudix enzymes represent an alternative class of hydrolases (including ADPrases) which, contrary to SVP, has been structurally and mechanistically characterized [2-4]. Our systematic screen of bacterial nudix hydrolases identified RppH from *Escherichia coli* as a pyrophosphatase capable of cleaving protein-conjugated ADPr to phosphoribose, albeit approximately 10x less efficiently than SVP (see Chapter 3). This activity could potentially be amplified by protein engineering, producing a robust tool for the reliable hydrolysis of every ADP-ribose polymer and monomer in a complex mixture to a single phosphoribose.

Engineering enzymes to modify substrate specificity or activity typically relies upon mutagenesis, either through rational design, directed evolution, or semi-rational design. The first, rational design, involves mutating discrete residues based on knowledge of the protein structure and catalytic mechanism [5]. The second, directed evolution, follows the process of natural selection in that it subjects a gene to iterative rounds of random mutation and selection [6]. As this process is random, knowledge of the protein structure or functional mechanism is not necessary, however a rapid screening process would be imperative to implementing this method, typically one which does not include purification of the expressed protein (the read-out can occur within cells or cell lysate). Semi-rational design exists as a compromise between rational design and directed evolution: critical regions of an enzyme (typically those surrounding the active site) are subjected to random mutagenesis and screened for the desired change in activity, thus obviating the need for a detailed mechanistic understanding of the protein function but making use of available structural information [7]. Considering the X-ray structure of substrate(RNA)-bound and free RppH has recently been solved [8] it is feasible to attempt rational design for the purpose of altering the substrate specificity of RppH, thus increasing the enzyme's pyrophosphatase activity against protein-conjugated ADPr.

An alternative to rational design for increasing RppH ADPr pyrophosphatase activity is to immobilize RppH to a matrix, a technique which has been shown to increase activity through buffering of local and global pH in a reaction mixture [9]. This buffering occurs due to decreased diffusion rates around the enzyme-conjugated matrix, allowing the researcher to equilibrate the matrix in the preferred pH for the enzyme prior to exposing the matrix to the substrate-compatible buffered reaction mixture. As enzymes often have a very narrow pH range for optimal activity (in the case of RppH the range is from pH 8.5-9.0 [10]) this buffering could be quite valuable for processing ADP-ribosylated substrates which must be kept at or below pH 7 in order to maintain all protein modifications (see Chapter 3).

Our screen began with the nudix ADPrases, as we rationalized that enzymes capable of hydrolyzing free ADPr would also be capable of degrading protein conjugated ADPr, a hypothesis

that we were unable to prove true (see Chapter 3). Structural analysis revealed that the active sites for these enzymes were highly restricted, largely due to their dimeric nature, in which both monomers contribute to substrate binding and specificity (see Figure 3-2). Circular permutation (the re-arrangement of protein domains, thus perturbing tertiary, and occasionally quaternary, structure [11]), then, could be a way to abolish dimerization of these enzymes (as has been done for the human protein β -Crystallin and the bacterial protein Cyanovirin-N [12, 13]), thus opening up the active site for protein conjugated ADPr.

5.2 Future Applications

5.2.1 Characterizing protein acceptors of ADP-ribose

Protein ADP-ribosylation has been implicated in a number of clinically and biologically important cellular pathways, several of which have been diagrammed in Figure 5-2. Identification of the ADP-ribosylation sites on these substrates has so far been laborious and time-consuming, often requiring multiple rounds of mutagenesis and activity assays to identify candidate conjugation sites. A successful example of this is the identification of E255, D256 and E268 on p53 as ADPr acceptor residues [14], site identification by mutagenesis is rarely so simple with ADP-ribosylation, however, as can be seen from similar attempts on PARP1 [15, 16] and BRCA1 [17]. Mass spectrometry based approaches from our group (this thesis) and Zhang et al. [1] have substantially increased the number of known protein ADP-ribosylation sites, though a brief comparison with Figure 5-2 reveals the non-saturating nature of these approaches: Zhang et al. identified E2043 as an ADP-ribosylation site on the tail of Numa1, our group identified K350 as an ADPr acceptor on HNRNPA1 (see Supplementary Figure 2-6 and Figure 5-3), all other substrates (including p53) were not characterized by either of these methods. Substrate characterization, then, may require a targeted approach (e.g. through the use of recombinant, tagged forms of the substrate of interest which can then be enriched) as well as site validation through mutagenesis.

5.2.2 Identifying critical changes in the ADP-ribosylated proteome

As covered in Chapter 1, PARP inhibitors are an important class of therapeutic drugs used (or undergoing clinical trial) for cancer treatment, with potential for application in: neurotrauma, neurodegenerative diseases, autoimmune diseases, and myocardial infarction (see Figures 1-4 and 1-5). As a molecularly targeted chemotherapeutic, it is rational to consider the activation state of the cellular processes affected by inhibition of PARP when selecting patients and tumor types as well as appropriate adjuvant and neoadjuvant therapies. Accordingly, researchers have long considered the state of DNA damage repair and overall PARylation levels when predicting PARP inhibitor sensitivity and/or choosing complementary therapies (see Figure 5-4). These conjectures are based on the knowledge that automodification of PARP1 and PARP2 (often considered the primary targets of PARP inhibitors) plays a role in DNA damage repair; therefore an increase in global PARylation is predictive of high levels of DNA damage repair and thus PARPi sensitivity. This overly simplistic model has guided PARP inhibitor development over the last ten years, ultimately resulting in FDA approval of the PARP inhibitor Olaparib for use by patients with BRCA mutant ovarian cancer [18]. Future development of these inhibitors, particularly outside of the realm of adjuvant DNA damage, will require a more precise understanding of cellular PARylation levels. For example, an increase in cellular PARylation could be due to changing levels of protein conjugated PAR or free PAR; elongation of existing PARylation sites or PARylation of new proteins. Distinguishing between these scenarios could separate out the effects exerted by unique PARP inhibitors (for example those which inhibit PARP5a and 5b versus those that inhibit PARP1 and 2), identifying cellular environments as appropriate targets. The pipeline described in this thesis has the potential to not only characterize the changing PARylation state at the level of the amino acid, but will also detect MARYlation, a PTM which is also regulated by PARP inhibitors but otherwise invisible to the ADPr antibodies currently available. This new understanding of protein ADP-ribosylation could allow researchers to recognize molecular patterns predictive of disease progression and PARPi sensitivity/resistance. Ultimately, robust biomarkers could be identified as a simple and reliable readout for clinical applications.

5.2.3 Searching for biomarkers of disease, treatment and drug resistance.

As discussed above, cells deficient in DNA damage repair (particularly homologous recombination) are considered good candidates for PARPi treatment, and this understanding has led to the large majority of PARPi clinical trials being designed by either (1) co-treating patients with DNA damaging agents such as ionizing radiation or alkylating agents or (2) enrolling patients with presumably defective DNA repair based on genetic analysis. The most successful genetic biomarkers employed in this scenario are BRCA1 and BRCA2, both critical components of homologous recombination that are part of the two-hit model of PARP sensitivity: losing BRCA and PARP together is much more cytotoxic than the loss of one alone. As biomarkers, however, BRCA mutations have limits: BRCA mutated tumors can acquire resistance to PARP inhibitors [19], patients with breast or ovarian cancer that lacks BRCA mutations can respond to PARP inhibitors [20, 21], and only 15% of high-grade epithelial ovarian cancers and 20% of triple-negative breast cancers (the two most PARPi responsive cancer types) carry BRCA mutations [22, 23]. There is a need, then, for a more robust marker for PARPi sensitivity. Considering the activity targeted by PARPi is the PTM ADP-ribosylation it is worth looking for ADP-ribosylated substrates in this context.

The observation of increased levels of PARP and PARylation has already prompted researchers to suggest PARPi for the treatment of non-cancerous disease states: PARP1 antibodies have been detected in the sera of patients with the autoimmune disorders: lupus, rheumatoid arthritis, scleroderma, Sjögren's syndrome, pulmonary fibrosis, sarcoid, ulcerative colitis, and Crohn's disease [24, 25], and general up-regulation of cellular protein PARylation has been detected in the neurodegenerative disorders Alzheimer's [26], amyotrophic lateral sclerosis [27] and Parkinson's (in which PARP1 expression is elevated [28]). As discussed in the previous section, however, broad observations of increased PARylation levels are poor indicators of molecular changes at the level of cellular pathways and processes. Identifying the contextual alterations in protein ADP-ribosylation with single amino acid resolution would allow researchers to begin the search for novel PARPi sensitivity biomarkers, as well as markers of disease progression.

5.3 Final Thoughts

The study of protein ADP-ribosylation has much to offer both basic and translational science: it is an intriguing PTM that manages to modify more amino acid residues than any other known PTM, it appears to have multiple means of regulating proteins through its highly charged polymeric presence, as well as what seems to sometimes be a regional (not site) specific protein modification. Despite over 50 years of research, scientists do not fully understand or agree upon the clinically and biologically significant mechanism of PARP1 automodification. Despite these challenges, great strides (and one great stumble [29]) have been made in the realm of chemotherapeutic PARP inhibitors, opening doors for real and significant impact on the lives of patients. In 2005 the ADP-ribosylation field learned that cancer cells harboring mutations in the breast cancer genes BRCA1 or BRCA2 were highly sensitive to PARP inhibition, less than 10 years later the first FDA approved PARP inhibitor appeared in cancer clinics for BRCA mutation positive ovarian cancer patients. Considering the vast progress that has been made recently in the study of ADP-ribosylation, not least the connections being made by our group and others to the mass spectrometry field, it is not unreasonable to look forward to the next great discovery that will propel PARP inhibitors forward, this time along with the insight gained from a fundamental understanding of the molecular and cellular implications of altering protein ADP-ribosylation.

5.4 Figures

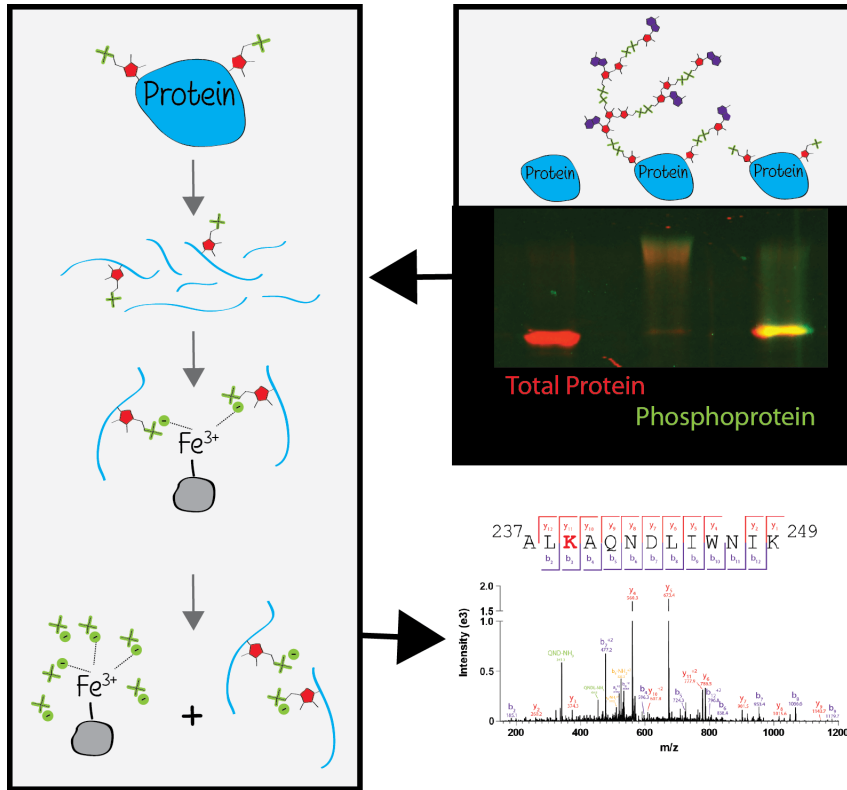


Figure 5-1 Pipeline for identifying ADP-ribosylation sites.

Protein-conjugated ADP-ribose (mono and poly) are hydrolyzed to phosphoribose, an SDS page gel shows the protein reaction occurring on a model substrate (total protein, in red, was visualized by coomassie blue staining, phosphoproteins, in green, were visualized using the Pro-Q Diamond kit from invitrogen). Phosphoribosylated protein is digested to peptides and phosphoribosylated peptides are enriched on a positively charged matrix (in this depiction, Iron(III) IMAC resin is used). Enriched peptides are competitively eluted by excess phosphate, allowing for target peptides to be identified by LC-MS/MS.

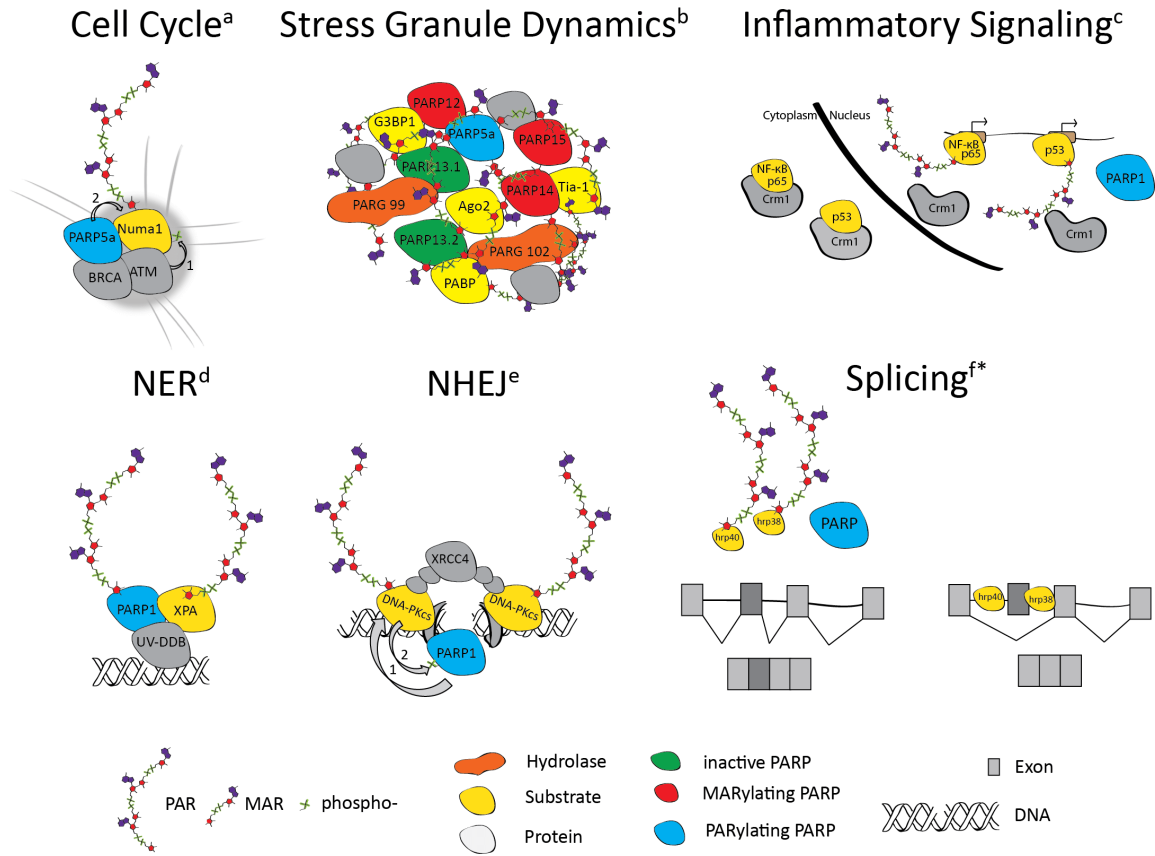


Figure 5-2 Snapshots showing protein ADP-ribosylation in cellular pathways.

NER = Nucleotide Excision Repair, NHEJ = Non-Homologous End Joining, PARP = Poly(ADP-ribose) Polymerase, PARG = Poly(ADP-ribose) Glycohydrolase, Numa1 = Nuclear mitotic apparatus protein 1, Crm1 = Exportin1/XPO1, NFκB = Nuclear Factor kappaB, XRCC = X-ray Repair Cross-Complementing Protein 4, UV-DDB = UV-damaged DNA Binding Protein, DNA-dependent Protein Kinase, catalytic subunit. *pathway observed in *Drosophila melanogaster*, hrp38's human homologue is HNRNPA1, hrp40 is an HNRNP protein (no direct homologue). *Drosophila* only has one PARP.

^a[30, 31]^b[32] ^c[14, 33] ^d[34]^e[35] ^f[36]

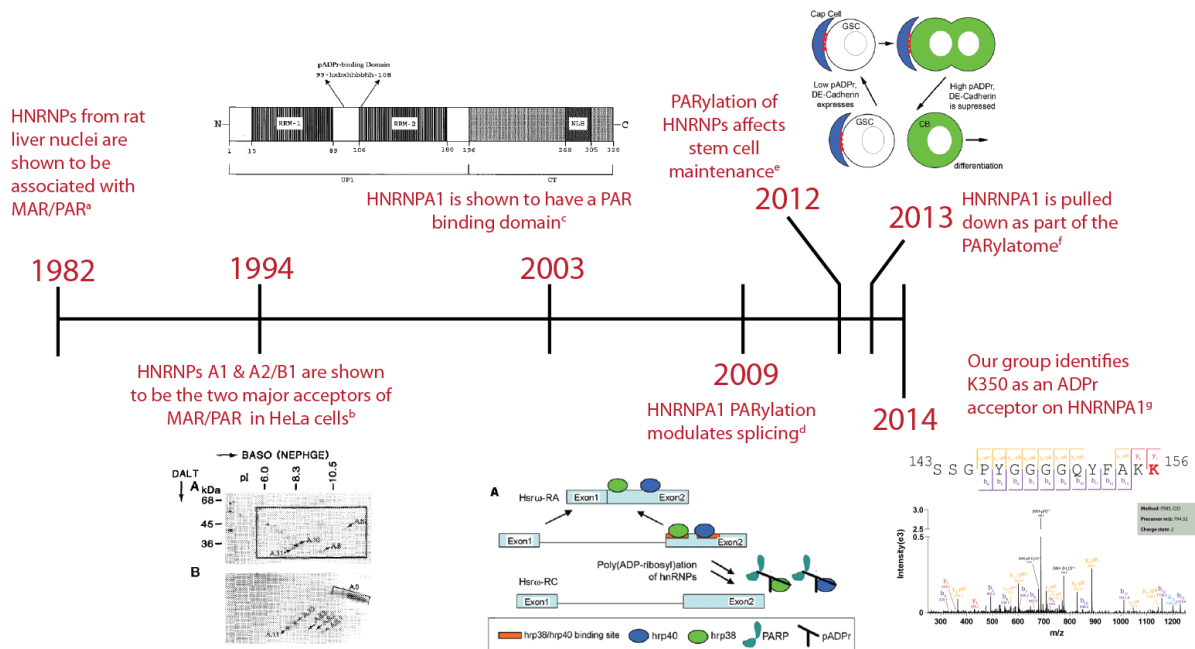


Figure 5-3 A historical perspective on research into ADP-ribosylation of HNRNPA1

We identified K350 as an endogenous ADP-ribosylation site on HNRNPA1 (see Chapter 2), adding to over 30 years of research into the ADP-ribosylation of HNRNPA1.

^a[37]^b[38]^c[39]^d[36]^e[40]^f[41, 42]^g[43]

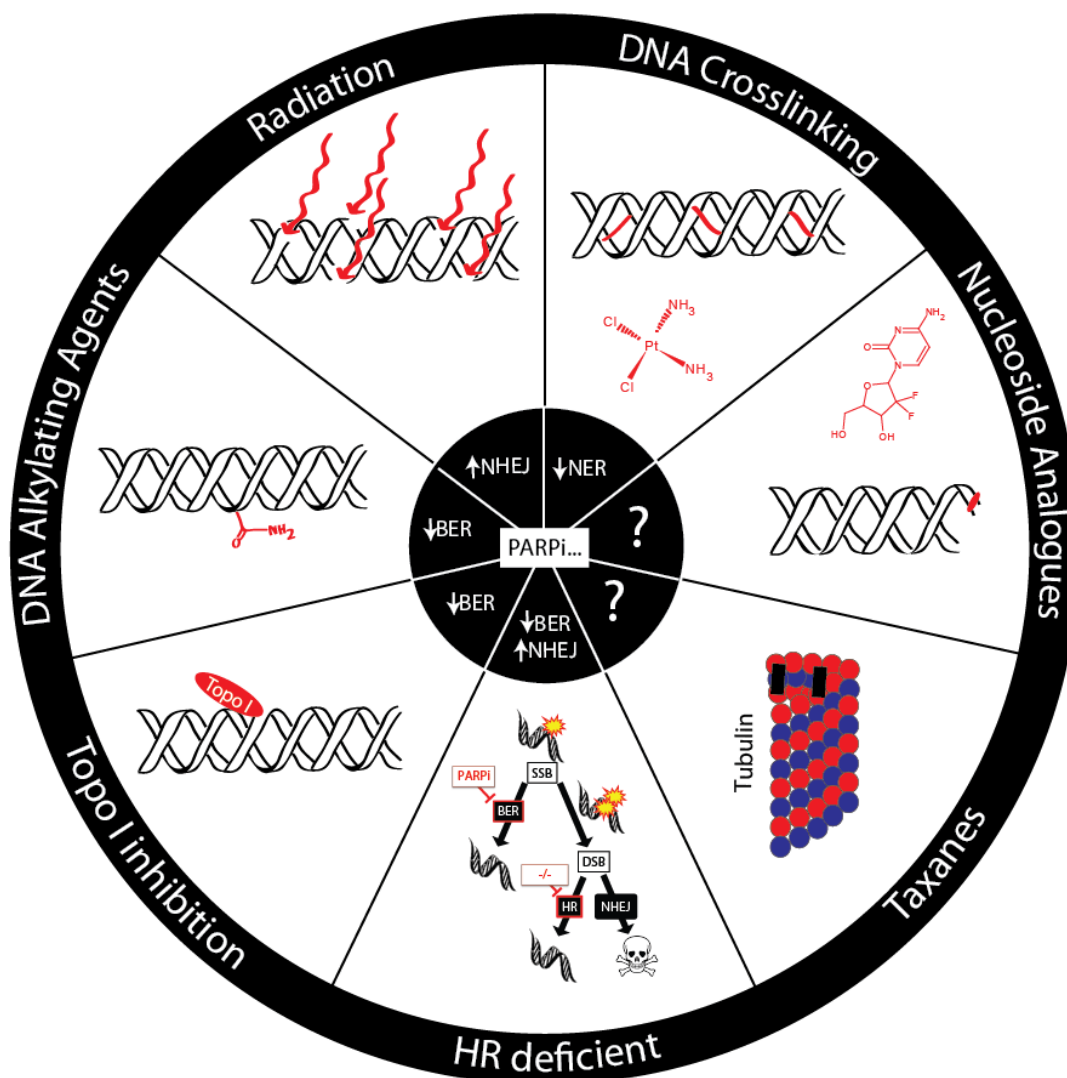


Figure 5-4 Combination therapies maximize PARPi potential as a chemotherapeutic agent.

Clinical trials are ongoing that employ combination therapies to enhance the cytotoxicity of PARP inhibitors. Most of these complementary therapies fall into one of seven categories: HRR deficient (the patient carries this status due to loss or mutation of an HRR protein, including the BRCA proteins)[44], Taxanes (which block mitosis through the suppression of microtubule dynamics)[45], Nucleoside Analogues (which are erroneously incorporated into DNA, blocking transcription) [46], DNA Crosslinking [47], Radiation [48, 49], DNA Alkylating Agents [50], or Topoisomerase I inhibition (wherein Topo I can no longer repair the nick it made while releasing torsional stress) [51]. For many of these therapies the benefits of PARP inhibition can be at least partly attributed to the

loss of the high-fidelity SSB repair pathways BER and NER or the stimulation of the low-fidelity DSB repair pathway NHEJ [52-54]. In the case of Taxanes and Nucleoside Analogues, however, DNA repair is not expected to play a direct role in their sensitization, leaving the mechanism of synergistic cytotoxicity unknown. BER = Base Excision Repair, HR = Homologous Recombination, NHEJ = Non-Homologous End Joining, SSB = Single Stranded Break, DSB = Double Stranded Break, PARPi = PARP inhibitor, Topo = Topoisomerase.

5.5 References

1. Zhang, Y., et al., *Site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome*. Nat Methods, 2013. **10**(10): p. 981-4.
2. Mildvan, A.S., et al., *Structures and mechanisms of Nudix hydrolases*. Arch Biochem Biophys, 2005. **433**(1): p. 129-43.
3. McLennan, A.G., *The Nudix hydrolase superfamily*. Cell Mol Life Sci, 2006. **63**(2): p. 123-43.
4. Bessman, M.J., D.N. Frick, and S.F. O'Handley, *The MutT proteins or "Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes*. J Biol Chem, 1996. **271**(41): p. 25059-62.
5. Cedrone, F., A. Menez, and E. Quemeneur, *Tailoring new enzyme functions by rational redesign*. Curr Opin Struct Biol, 2000. **10**(4): p. 405-10.
6. Tracewell, C.A. and F.H. Arnold, *Directed enzyme evolution: climbing fitness peaks one amino acid at a time*. Curr Opin Chem Biol, 2009. **13**(1): p. 3-9.
7. Chica, R.A., N. Doucet, and J.N. Pelletier, *Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design*. Curr Opin Biotechnol, 2005. **16**(4): p. 378-84.
8. Vasilyev, N. and A. Serganov, *Structures of RNA Complexes with the Escherichia coli RNA Pyrophosphohydrolase RppH Unveil the Basis for Specific 5'-End-dependent mRNA Decay*. J Biol Chem, 2015. **290**(15): p. 9487-99.
9. Rodrigues, R.C., et al., *Modifying enzyme activity and selectivity by immobilization*. Chem Soc Rev, 2013. **42**(15): p. 6290-307.
10. Bessman, M.J., et al., *The gene ygdP, associated with the invasiveness of Escherichia coli K1, designates a Nudix hydrolase, Orf176, active on adenosine (5')-pentaphospho-(5')-adenosine (Ap5A)*. J Biol Chem, 2001. **276**(41): p. 37834-8.
11. Yu, Y. and S. Lutz, *Circular permutation: a different way to engineer enzyme structure and function*. Trends Biotechnol, 2011. **29**(1): p. 18-25.
12. Wright, G., et al., *Circular permutation of betaB2-crystallin changes the hierarchy of domain assembly*. Protein Sci, 1998. **7**(6): p. 1280-5.
13. Barrientos, L.G., et al., *Solution structure of a circular-permuted variant of the potent HIV-inactivating protein cyanovirin-N: structural basis for protein stability and oligosaccharide interaction*. J Mol Biol, 2003. **325**(1): p. 211-23.
14. Kanai, M., et al., *Inhibition of Crm1-p53 interaction and nuclear export of p53 by poly(ADP-ribosylation)*. Nat Cell Biol, 2007. **9**(10): p. 1175-83.

15. Tao, Z., P. Gao, and H.W. Liu, *Identification of the ADP-ribosylation sites in the PARP-1 automodification domain: analysis and implications*. J Am Chem Soc, 2009. **131**(40): p. 14258-60.
16. Altmeyer, M., et al., *Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites*. Nucleic Acids Res, 2009. **37**(11): p. 3723-38.
17. Hu, Y., et al., *PARP1-driven Poly-ADP-ribosylation Regulates BRCA1 Function in Homologous Recombination Mediated DNA Repair*. Cancer Discov, 2014.
18. Deeks, E.D., *Olaparib: first global approval*. Drugs, 2015. **75**(2): p. 231-40.
19. Bouwman, P. and J. Jonkers, *Molecular pathways: how can BRCA-mutated tumors become resistant to PARP inhibitors?* Clin Cancer Res, 2014. **20**(3): p. 540-7.
20. Gelmon, K.A., et al., *Olaparib in patients with recurrent high-grade serous or poorly differentiated ovarian carcinoma or triple-negative breast cancer: a phase 2, multicentre, open-label, non-randomised study*. Lancet Oncol, 2011. **12**(9): p. 852-61.
21. Ledermann, J., et al., *Olaparib maintenance therapy in platinum-sensitive relapsed ovarian cancer*. N Engl J Med, 2012. **366**(15): p. 1382-92.
22. Gonzalez-Angulo, A.M., et al., *Incidence and outcome of BRCA mutations in unselected patients with triple receptor-negative breast cancer*. Clin Cancer Res, 2011. **17**(5): p. 1082-9.
23. *Integrated genomic analyses of ovarian carcinoma*. Nature, 2011. **474**(7353): p. 609-15.
24. Negri, C., et al., *Autoantibodies to poly(ADP-ribose)polymerase in autoimmune diseases*. Autoimmunity, 1990. **6**(3): p. 203-9.
25. Reumaux, D., et al., *Distinct production of autoantibodies to nuclear components in ulcerative colitis and in Crohn's disease*. Clin Immunol Immunopathol, 1995. **77**(3): p. 349-57.
26. Love, S., R. Barber, and G.K. Wilcock, *Increased poly(ADP-ribosyl)ation of nuclear proteins in Alzheimer's disease*. Brain, 1999. **122** (Pt 2): p. 247-53.
27. Kim, S.H., et al., *PARP expression is increased in astrocytes but decreased in motor neurons in the spinal cord of sporadic ALS patients*. J Neuropathol Exp Neurol, 2003. **62**(1): p. 88-103.
28. Soos, J., et al., *The expression of PARP, NF-kappa B and parvalbumin is increased in Parkinson disease*. Neuroreport, 2004. **15**(11): p. 1715-8.
29. O'Shaughnessy, J., et al., *Iniparib plus chemotherapy in metastatic triple-negative breast cancer*. N Engl J Med, 2011. **364**(3): p. 205-14.
30. Palazzo, L., et al., *ATM controls proper mitotic spindle structure*. Cell Cycle, 2014. **13**(7): p. 1091-100.

31. Lan, J., et al., *The 68-kDa telomeric repeat binding factor 1 (TRF1)-associated protein (TAP68) interacts with and recruits TRF1 to the spindle pole during mitosis*. J Biol Chem, 2014. **289**(20): p. 14145-56.
32. Leung, A.K., et al., *Poly(ADP-ribose) regulates stress responses and microRNA activity in the cytoplasm*. Mol Cell, 2011. **42**(4): p. 489-99.
33. Kameoka, M., et al., *Evidence for regulation of NF-kappaB by poly(ADP-ribose) polymerase*. Biochem J, 2000. **346 Pt 3**: p. 641-9.
34. Fischer, J.M., et al., *Poly(ADP-ribose)-mediated interplay of XPA and PARP1 leads to reciprocal regulation of protein function*. FEBS J, 2014. **281**(16): p. 3625-41.
35. Ruscetti, T., et al., *Stimulation of the DNA-dependent protein kinase by poly(ADP-ribose) polymerase*. J Biol Chem, 1998. **273**(23): p. 14461-7.
36. Ji, Y. and A.V. Tulin, *Poly(ADP-ribosyl)ation of heterogeneous nuclear ribonucleoproteins modulates splicing*. Nucleic Acids Res, 2009. **37**(11): p. 3501-13.
37. Kostka, G. and A. Schweiger, *ADP-ribosylation of proteins associated with heterogeneous nuclear RNA in rat liver nuclei*. Biochim Biophys Acta, 1982. **696**(2): p. 139-44.
38. Prasad, S., J. Walent, and A. Dritschilo, *ADP-ribosylation of heterogeneous ribonucleoproteins in HeLa cells*. Biochem Biophys Res Commun, 1994. **204**(2): p. 772-9.
39. Gagne, J.P., et al., *A proteomic approach to the identification of heterogeneous nuclear ribonucleoproteins as a new family of poly(ADP-ribose)-binding proteins*. Biochem J, 2003. **371**(Pt 2): p. 331-40.
40. Ji, Y. and A.V. Tulin, *Poly(ADP-ribose) controls DE-cadherin-dependent stem cell maintenance and oocyte localization*. Nat Commun, 2012. **3**: p. 760.
41. Gagne, J.P., et al., *Quantitative proteomics profiling of the poly(ADP-ribose)-related response to genotoxic stress*. Nucleic Acids Res, 2012. **40**(16): p. 7788-805.
42. Jungmichel, S., et al., *Proteome-wide Identification of Poly(ADP-Ribosyl)ation Targets in Different Genotoxic Stress Responses*. Mol Cell, 2013.
43. Daniels, C.M., S.E. Ong, and A.K. Leung, *A phosphoproteomic approach to characterize protein mono and poly(ADP-ribosyl)ation sites from whole cell lysate*. J Proteome Res, 2014.
44. Curtin, N.J. and C. Szabo, *Therapeutic applications of PARP inhibitors: anticancer therapy and beyond*. Mol Aspects Med, 2013. **34**(6): p. 1217-56.
45. McGrogan, B.T., et al., *Taxanes, microtubules and chemoresistant breast cancer*. Biochim Biophys Acta, 2008. **1785**(2): p. 96-132.

46. Ueno, H., K. Kiyosawa, and N. Kaniwa, *Pharmacogenomics of gemcitabine: can genetic studies lead to tailor-made therapy?* Br J Cancer, 2007. **97**(2): p. 145-51.
47. McHugh, P.J., V.J. Spanswick, and J.A. Hartley, *Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance.* Lancet Oncol, 2001. **2**(8): p. 483-90.
48. Godon, C., et al., *PARP inhibition versus PARP-1 silencing: different outcomes in terms of single-strand break repair and radiation susceptibility.* Nucleic Acids Res, 2008. **36**(13): p. 4454-64.
49. Willers, H., J. Dahm-Daphi, and S.N. Powell, *Repair of radiation damage to DNA.* Br J Cancer, 2004. **90**(7): p. 1297-301.
50. Drablos, F., et al., *Alkylation damage in DNA and RNA--repair mechanisms and medical significance.* DNA Repair (Amst), 2004. **3**(11): p. 1389-407.
51. Pommier, Y., *Topoisomerase I inhibitors: camptothecins and beyond.* Nat Rev Cancer, 2006. **6**(10): p. 789-802.
52. Dantzer, F., et al., *Base excision repair is impaired in mammalian cells lacking Poly(ADP-ribose) polymerase-1.* Biochemistry, 2000. **39**(25): p. 7559-69.
53. Patel, A.G., J.N. Sarkaria, and S.H. Kaufmann, *Nonhomologous end joining drives poly(ADP-ribose) polymerase (PARP) inhibitor lethality in homologous recombination-deficient cells.* Proc Natl Acad Sci U S A, 2011. **108**(8): p. 3406-11.
54. Robu, M., et al., *Role of poly(ADP-ribose) polymerase-1 in the removal of UV-induced DNA lesions by nucleotide excision repair.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. 1658-63.

Appendices

6 Characterizing phosphorylation on MacroD2

6.1 Abstract

In an effort to develop the protein MacroD2 as a tool for distinguishing between sites of mono- and poly(ADP-ribose) (see chapter 4) we have identified a series of phosphorylation sites on the C-terminus of MacroD2, a protein which was otherwise not known to be phosphorylated. The site stoichiometry of these phosphorylation sites is increased on mutant forms of MacroD2 which are unable to bind and/or hydrolyze ADPr, suggesting a functional role for phosphorylation on MacroD2. Further studies are warranted to determine the roles these phosphorylation sites place in MacroD2 localization, function and cellular role.

6.2 Introduction

Complete characterization of a protein ADP-ribosylation event will ultimately require knowledge of the amino acid conjugation site, the occupancy of this conjugation site, and the structure of the modification itself (mono/poly, linear/branched) – an idea presented and defended in Chapter 4 of this thesis. To this end, we have proposed using ADP-ribose binding proteins (or their domains) to distinguish between mono- and poly(ADP-ribosyl)ated proteins by way of their specificity for binding and/or removing one, but not the other, of these modifications. In our analysis of all ADPr binding domains (see Table 3-1) macroD2 stood out to us as a potential tool for enriching mono-, but not poly-, ADP-ribosylated proteins and/or peptides for identification by mass spectrometry. Specifically, this enzyme was shown to bind and remove mono(ADP-ribose) from acidic residues, activities which could be modulated by the introduction of single point mutations [1] – these mutations would thus allow researchers to robustly distinguish between MacroD2 interaction partners which are dependent upon the macrodomain binding to ADPr and those which are interacting with MacroD2 independent of its ADPr-binding abilities. In this appendix we have begun

to characterize MacroD2 and its functionally deficient mutants (binding and/or hydrolase deficient) for the purpose of their application as tools for distinguishing between sites of mono- and poly(ADP-ribose). Serendipitously, however, we have found a swath of phosphorylation sites on the C-terminus of full-length macroD2, a protein which was not previously known to be phosphorylated. Furthermore, we have shown that phosphorylation site occupancy (relative to the unoccupied, ie unmodified, state of the amino acid site) increases for the ADPr binding and/or hydrolase deficient mutants of macroD2 as compared to the wildtype.

6.3 Materials and Methods

Expression and purification of HisPARP-1

See section 2.3.

Purification of Snake Venom Phosphodiesterase I (SVP)

See section 2.3. Snake venom phosphodiesterase was obtained from United States Biological, catalog number P4072, lot number L14030507 C14062702.

Preparing oligos for in vitro PARP-1 activation

See section 2.3.

Automodification of E988Q HisPARP-1 *in vitro*.

See section 2.3.

Cell Culture

HeLa cells were grown in the presence of heavy-isotope labeled arginine and lysine before PAR induction by N-methyl-N'-nitro-N-nitrosoguanidine, as in Chapter 2, page (update). Freestyle 293-F cells (Invitrogen) were cultured per manufacturer's recommendations. GFP-MacroD2 (as obtained from Dr. Andreas Ladurner[2]) was transfected into Freestyle 293-F cells using Lipofectamine-2000 following the manufacturer's protocol (1 ug of construct per 1e6 cells).

Immunoprecipitation of GFP-MacroD2

GFP-MacroD2 transfected 293-F cells were washed with ice-cold PBS and lysed in 1x mRIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% n-Octyl Glucoside, 1 mM EDTA, 1x SigmaFast Protease

inhibitor, and 0.1% sodium deoxycholate) on ice for 10 minutes. Lysate was cleared by centrifugation and added to pre-equilibrated GFP-Trap beads (from ChromoTek) in mRIPA buffer, 80 uL of slurry/65 mL of cells.

Affinity purification of mono(ADP-ribosyl)ated substrates

20 µg of GFP-MacroD2 (wildtype, binding mutant G100E or hydrolase mutant G100E/I189R/Y190N) on GFP-Trap beads was exposed to 5 µg of automodified PARP-1 E988Q and 500 µg of heavy-labeled, DNA damaged HeLa whole cell lysate in wash buffer (1M Urea, 200 mM Tris pH 7, 150 mM NaCl, 15 mM MgCl₂, 1% n-Octyl Glucoside) for 30 minutes at room temperature. Samples were washed three times with wash buffer and denatured in 8M Urea (in 50 mM Tris pH 7) at 37°C for 10 minutes before being reduced in 1 mM Tris-(2-Carboxyethyl)phosphine (Life Technologies) for 10 minutes and then alkylated in 2 mM 2-chloroacetamide (Sigma) for 10 minutes in the dark.

In-solution protein digestion

See section 2.3.

Phosphoenriching phosphorylated, ADP-ribosylated and phospho(ribosyl)ated peptides

See section 2.3.

NanoLC-MS/MS analysis

See section 2.3.

Database search of MS/MS spectra for peptide & protein identification

See section 2.3, with the following changes: MaxQuant version 1.5.2.8, human Uniprot FASTA database updated Dec. 2nd, 2014.

6.4 Results

Identification of the wildtype and mutant interactomes

GFP-MacroD2 wildtype as well as binding and hydrolase mutants were expressed and purified from human 293-F cells. As shown previously[1], the point mutation G100E abolishes ADPr binding and hydrolysis (hereafter referred to as the ‘binding mutant’), while the point

mutations I189R and Y190N together restore ADPr binding without restoring ADPr hydrolysis, thus providing the 'hydrolase mutant' used in this study (Figure 6-1). These proteins were then exposed to heavy (K_8R_{10}) labeled HeLa cell lysate to identify the unique interaction network accompanying each protein. Stable interactors, or those which were maintained from the GFP-IP, were distinguished by their light (K_0R_0) labeling state, while those newly acquired from the HeLa cell lysate carried heavy-labeled amino acids (see Figure 6-2). Mono(ADP-ribosyl)ated (MARylated) human PARP-1 E988Q was included as an ADPr-conjugated protein standard to ascertain the relative ADPr binding and hydrolase activity of the MacroD2 mutants with respect to wildtype. ADPr site identification was aided through the digestion of ADPr to phosphoribose by the enzyme snake venom phosphodiesterase and the subsequent enrichment of phospho(ribosyl)ated as well as phosphorylated peptides by immobilized metal affinity chromatography (IMAC) (for a detailed explanation of this pipeline see Chapter 2). Unfortunately no quality spectra showing PARP1 E988Q ADPr or phosphoribose sites were identified, and therefore ADPr enrichment and site identification by this method requires further study and will not be considered for this thesis appendix.

MacroD2 is highly phosphorylated and this phosphorylation is affected by modulation of ADPr-binding and hydrolase activities

Following GFP-MacroD2 exposure to heavy-labeled HeLa whole cell lysate the wildtype and mutant proteins were digested to peptides and phosphopeptides and phosphoenriched by IMAC. This phosphoenrichment produced four phosphopeptides from MacroD2 (Figure 6-3, panel A) which were identified in multiple states, including the unmodified forms found in the flowthrough (Figure 6-3 panel B). Both the phosphorylated and nonphosphorylated forms of the peptides occasionally showed methionine oxidation, a post-translational modification which is often seen as an artifact attributed to mass spectrometry sample processing, and will be considered such in this case (Figure 6-3 panel B). In order to examine the change in phosphorylation state between the wildtype and mutant forms of MacroD2 the phosphorylation occupancy of each phosphopeptide was compared, as in Figure 6-3 panel C. Briefly, the total ion current (TIC) read out from an LC-MS

analysis includes looking at the m/z ratios of all eluting peptides along with their corresponding intensities and retention times. In order to compare intensities of a single peptide across runs, however, the TIC is filtered to only display the m/z ratio of the peptide of interest, this is referred to as an extracted ion current, or XIC, and is shown in Figure 6-3 panel C for phosphopeptide #1 in this study. The intensities of these peaks are derived from the area under the curves and are considered an indication of the abundance of the peptide of interest (which is definitively identified through MS/MS fragmentation, known retention time, and the m/z ratio of the intact peptide). In this way the abundance of each form of the peptides of interest is determined and these abundances are compared across samples to ascertain changes in phosphorylation occupancy on the peptide of interest, the result of which is shown in Figure 6-3 panels D-H for phosphopeptides 1-4. In the case of all four peptides the phosphorylation occupancy increases for the ADPr binding and hydrolase mutants as compared to wildtype.

MacroD2 may be phosphorylated by Casein Kinase 1 α

As phosphorylation occupancy increased for the peptides of interest from both ADPr binding and ADPr hydrolysis deficient MacroD2 mutants we hypothesized that the increased phosphorylation may be a result of increased interaction with a protein kinase by the mutant proteins as compared to the wildtype protein. Accordingly we examined those proteins which were identified as interactors for the mutant proteins while not identified in the wildtype MacroD2 interactome (Figure 6-4 panel A). Of the serine/threonine kinases identified (cyclin-dependent kinase 2, casein kinase 1 α , integrin-linked protein kinase) only one, casein kinase 1 α , was found to have a target motif matching the MacroD2 phosphorylation sites identified, specifically either [pS/pT]X₁₋₂[S/T] or [D/E]_nX₁₋₂[S/T] (Figure 6-4 panel B)[3, 4].

6.5 Discussion

This pilot study has provided evidence that phosphorylation may play a role in MacroD2 function, and most importantly has identified novel phosphorylation sites on MacroD2, including: S276/T284/T285 (ambiguous), T390, S400, S401, T406, S415, T424 and S426 (see supplementary

spectra). The functional role of these phosphorylation events has yet to be determined, and should be addressed in future studies. Considering the variation in phosphorylation states between wildtype and mutant MacroD2 proteins it may be best to use the macrodomain of MacroD2 as bait for ADP-ribosylated proteins, thus removing the bias these phosphorylation events may impose between wildtype and mutant forms of MacroD2.

6.6 Acknowledgements

I would like to thank Drs Anthony Leung and Shao-En Ong for their guidance and financial support of this project, as well as Robert McPherson, a fellow member of the Leung Lab, for his assistance mutating, expressing, and purifying the GFP-MacroD2 proteins used as bait in this study. I would also like to thank Dr. Andreas Ladurner for kindly providing the MacroD2 construct used in this study.

6.7 Figures

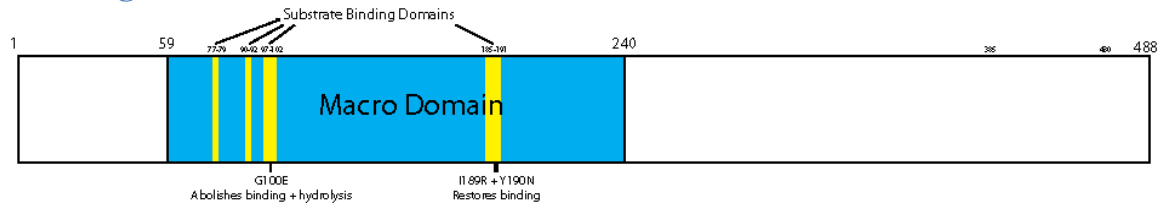


Figure 6-1 MacroD2 mutations modulate ADPr binding and hydrolysis

As discovered by Jankevicius et al in 2013[1], the MacroD2 point mutation G100E abolishes ADPr binding and hydrolysis by MacroD2, while the I189R and Y190N mutations (in combination) subsequently restore ADPr binding without restoring hydrolysis.

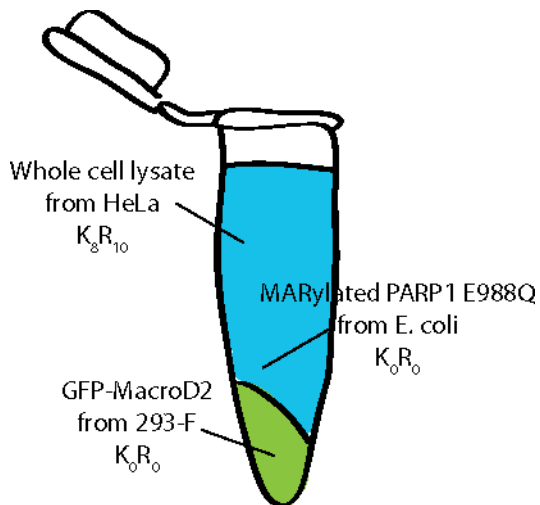


Figure 6-2 Acquiring the interaction network of MacroD2 wildtype and mutant proteins.

GFP-MacroD2 proteins were expressed and purified from human light-labeled 293-F cells before being used as bait against a mixture of whole cell lysate (heavy, human, HeLa cells) and *in vitro* MARylated human PARP1 E988Q (expressed and purified from E. coli cells, light).

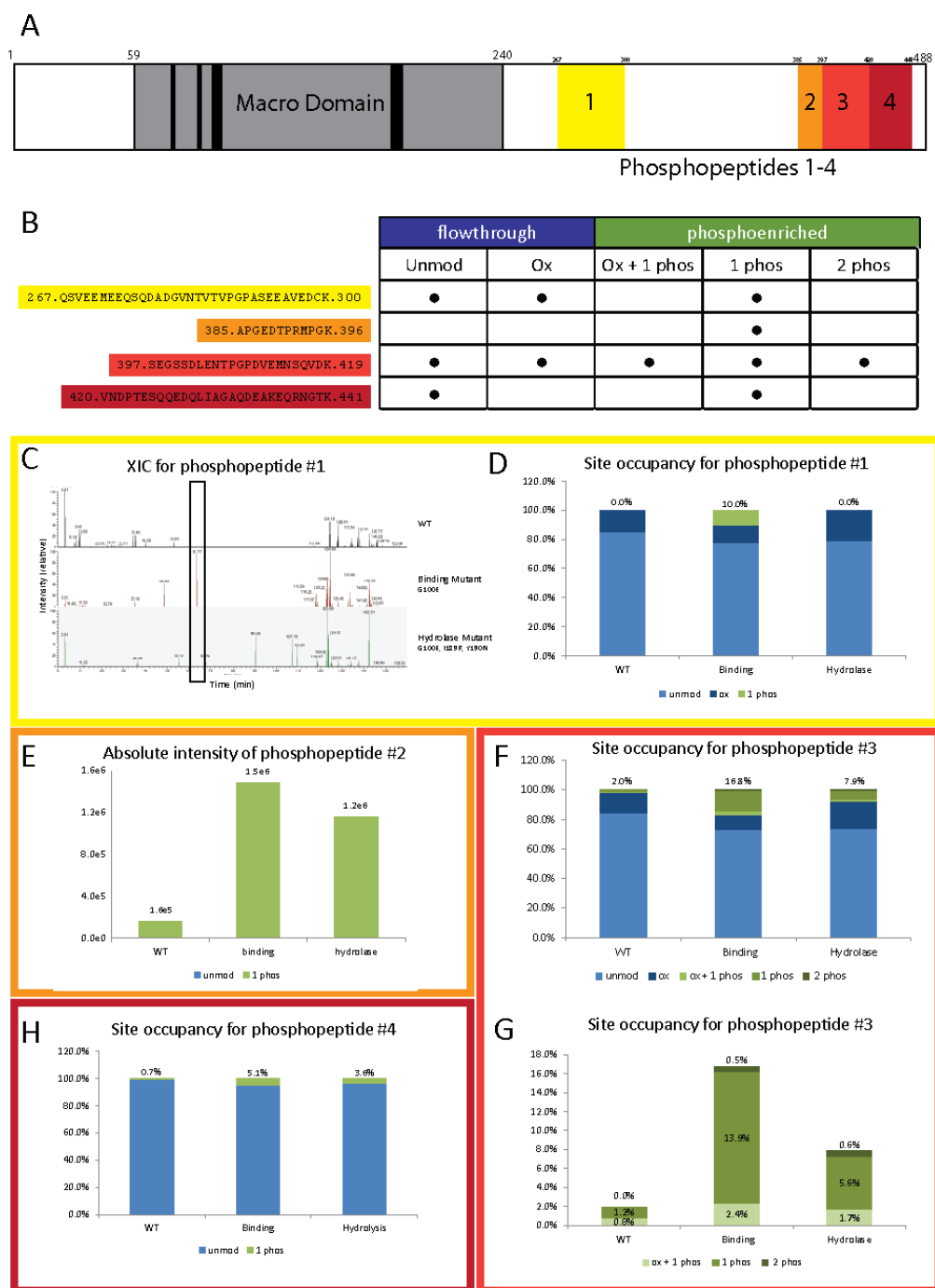


Figure 6-3 Binding and hydrolase deficient mutants of MacroD2 are more highly phosphorylated than wildtype.

Analysis by mass spectrometry revealed four phosphopeptides from MacroD2, labeled here as phosphopeptides 1-4 (A), three of which were found in multiple post-translationally modified states (B). The extracted ion current (XIC) for each peptidic form was collected as in (C) and plotted as the percent total intensity for each peptide, ie the phosphorylation site occupancy (D-H). In the case of

phosphopeptide #2 (E) the only form identified was singly phosphorylated, and therefore the absolute (instead of relative) intensity is shown.

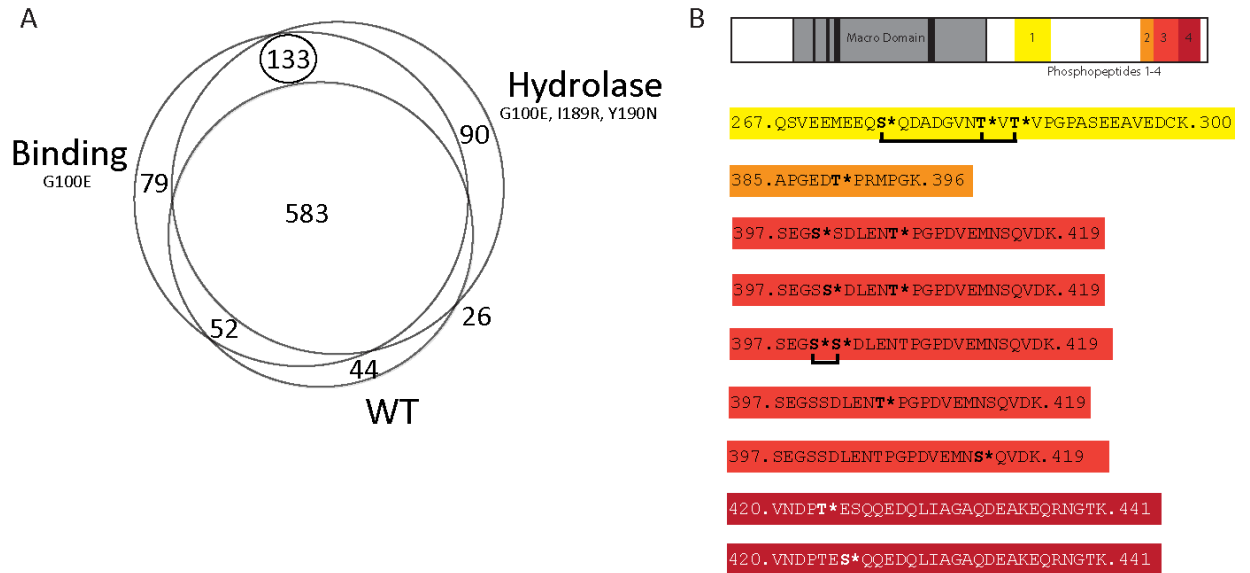
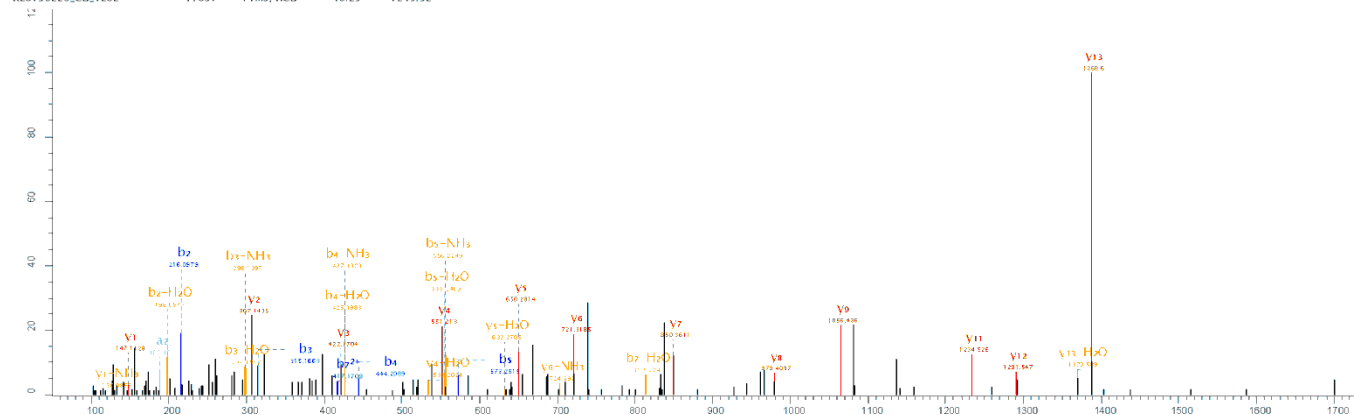


Figure 6-4 Casein Kinase 1 α may be responsible for the increased phosphorylation of the mutant MacroD2 proteins.

In order to procure a list of potential kinases responsible for the change in phosphorylation of MacroD2 the protein interactors were identified, particularly the kinases found to be enriched by the mutant, but not wildtype, forms of MacroD2 (A). The two bona fide Serine/Threonine kinases identified in this group were casein kinase 1 α (CK1 α) and cyclin-dependent kinase 2 (cdk2). The consensus motif for CK1 α most closely matched the sequences found in phosphopeptides 1-4: [pS/pT/D/E]X₁₋₂[S*/T*] (B).

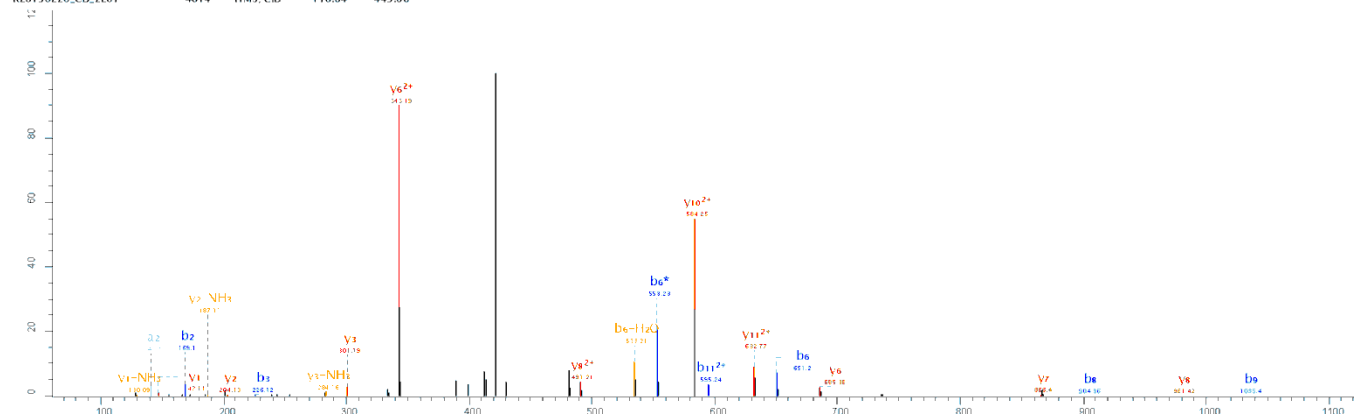
6.8 Supplementary Spectra

Raw file: R20150220_CD_1E02 Scan: 11037 Method: FTMS, HCD Score: 40.29 m/z: 1249.52



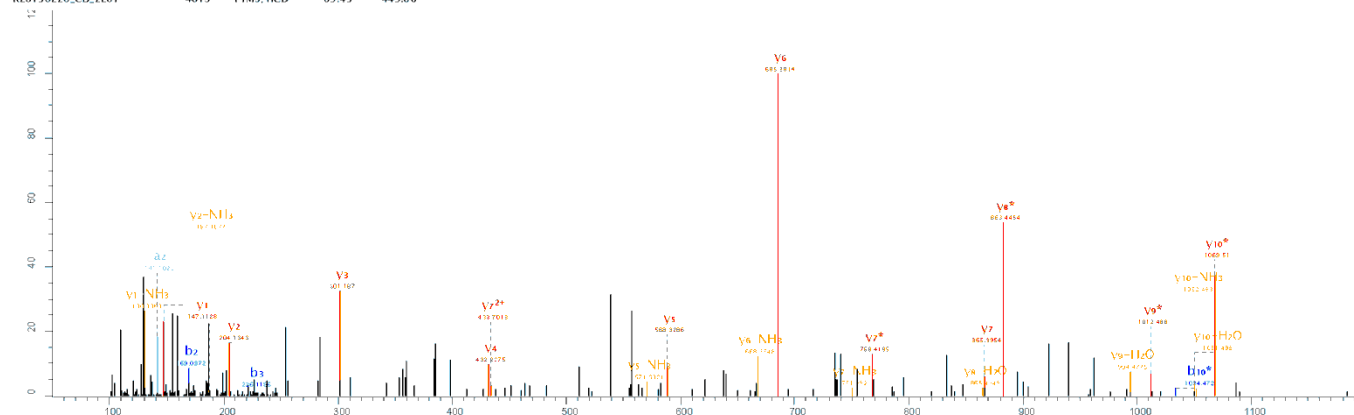
Sequence: - Q S V E E M E E Q S Q D A D G V N T V T V P G P A S E E A V E D C K -

Raw file: R20150220_CD_2E01 Scan: 4014 Method: ITMS, CID Score: 110.84 m/z: 445.86



Sequence: - A P G E D P R M P G K -

Raw file: R20150220_CD_2E01 Scan: 4013 Method: FTMS, HCD Score: 85.45 m/z: 445.86



Sequence: - A P G E D P R M P G K -

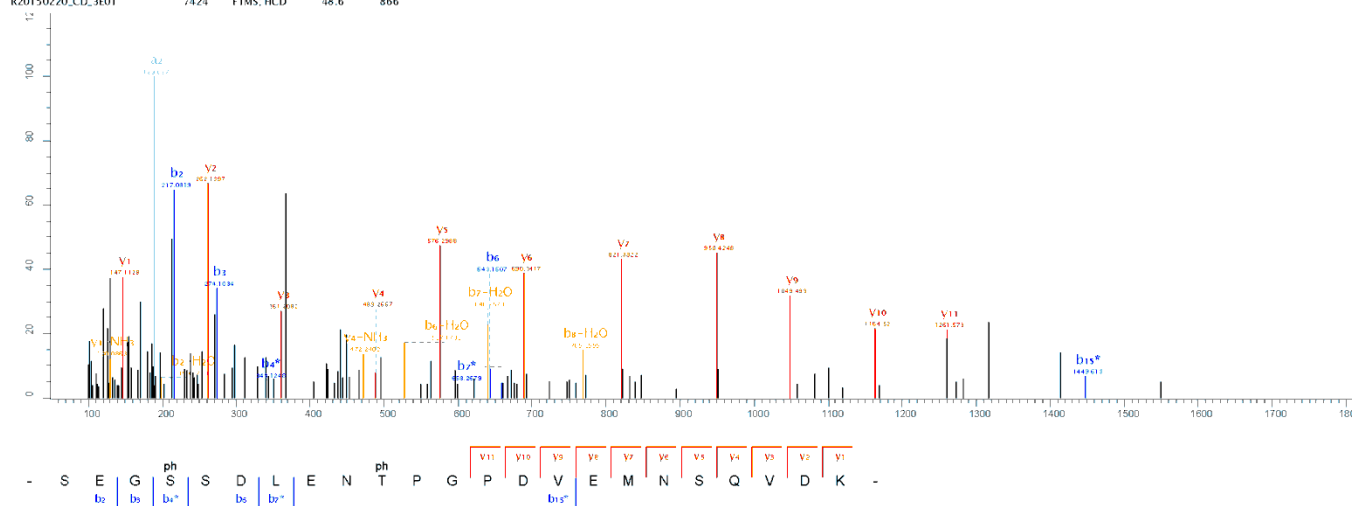
Raw file
R20150220_CD_3E01

Scan
7424

Method
FTMS, HCD

Score
48.6

m/z
866



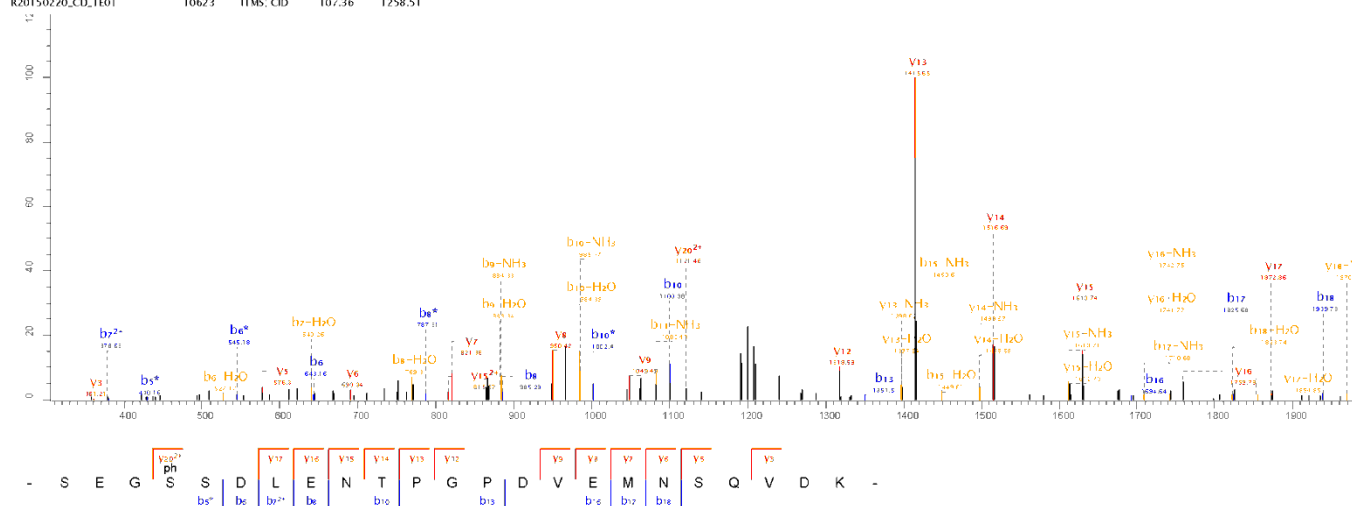
Raw file
R20150220_CD_1E01

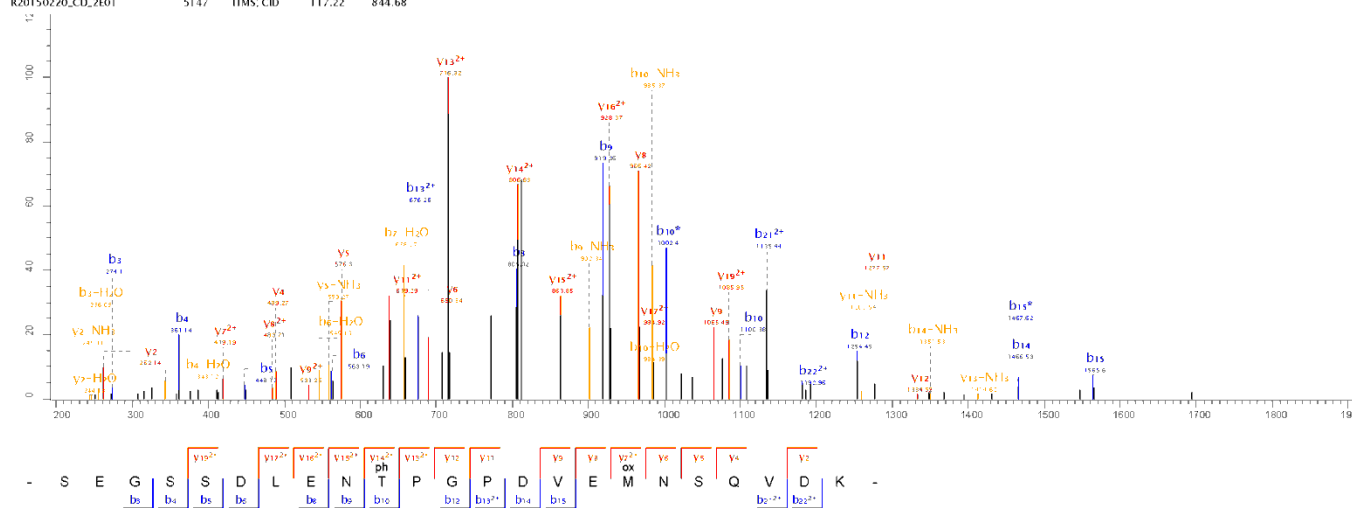
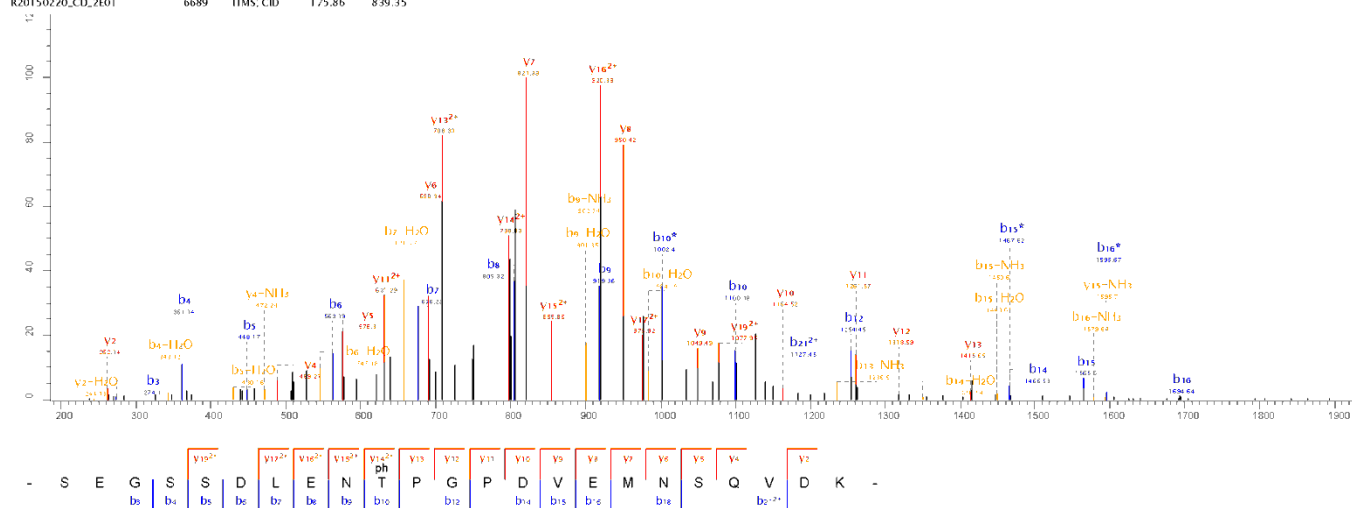
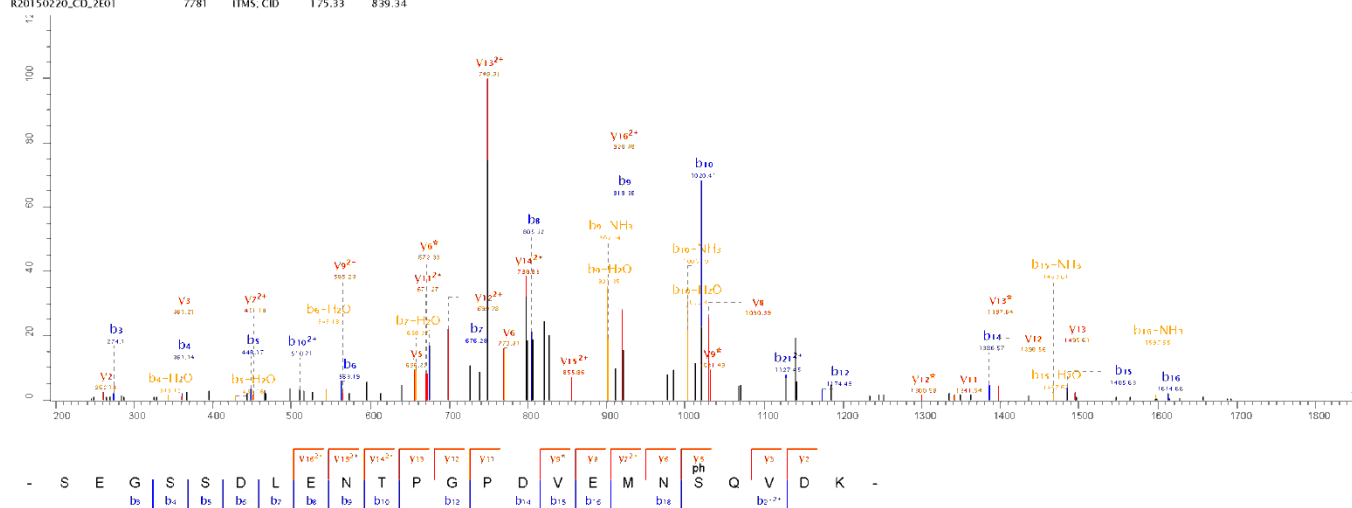
Scan
10623

Method
ITMS, CID

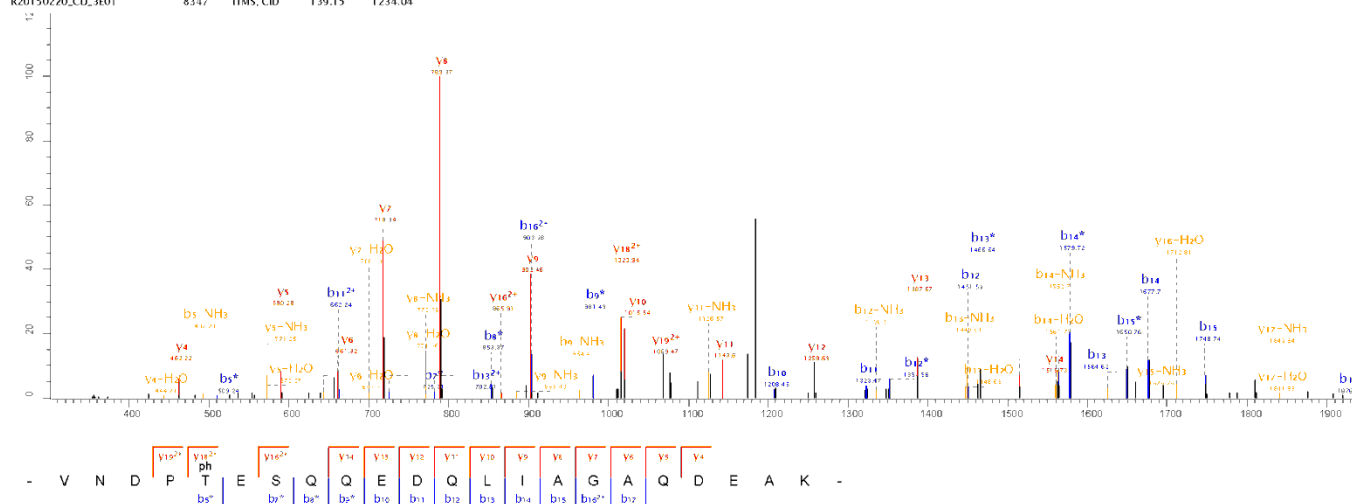
Score
107.36

m/z
1258.51

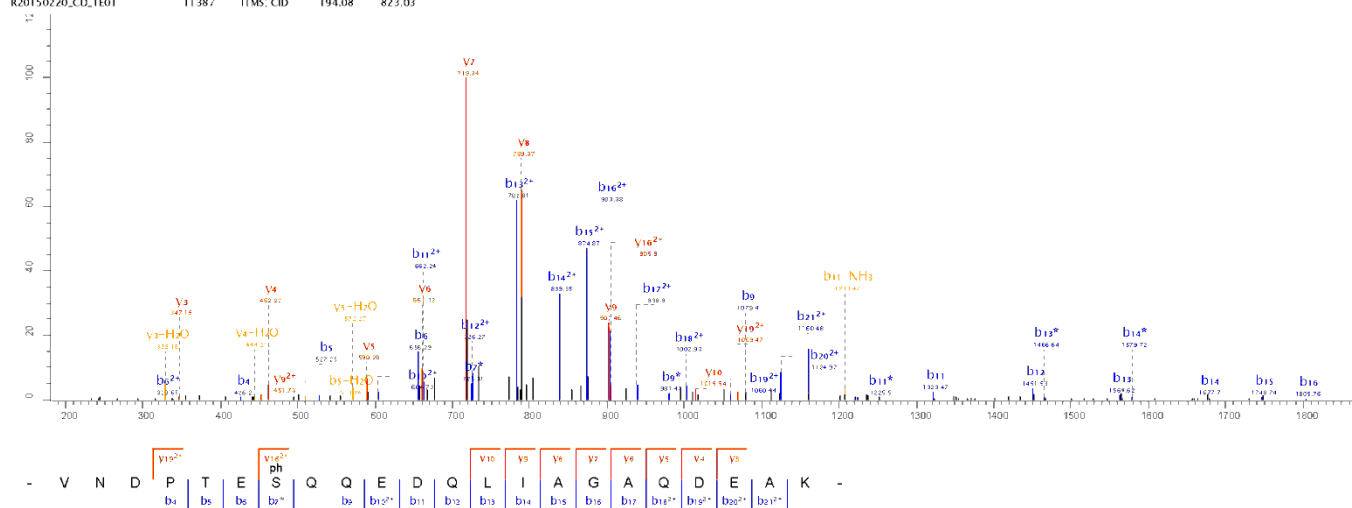




Raw file Scan Method Score m/z
R20150220_CD_3E01 8347 ITMS, CID 139.15 1234.04



Raw file Scan Method Score m/z
R20150220_CD_1E01 11387 ITMS, CID 194.08 823.03



6.9 References

1. Jankevicius, G., et al., *A family of macrodomain proteins reverses cellular mono-ADP-ribosylation*. Nat Struct Mol Biol, 2013. **20**(4): p. 508-14.
2. Timinszky, G., et al., *A macrodomain-containing histone rearranges chromatin upon sensing PARP1 activation*. Nat Struct Mol Biol, 2009. **16**(9): p. 923-9.
3. Flotow, H., et al., *Phosphate groups as substrate determinants for casein kinase I action*. J Biol Chem, 1990. **265**(24): p. 14264-9.
4. Flotow, H. and P.J. Roach, *Role of acidic residues as substrate determinants for casein kinase I*. J Biol Chem, 1991. **266**(6): p. 3724-7.

Curriculum Vitae

Primary Contact Info:
cs.dnls@gmail.com
17801 Bowie Mill Road
Derwood, MD 20855
cell: (717) 451 – 6367

Casey M. Daniels

Johns Hopkins Bloomberg
School of Public Health
cmdaniel@jhsph.edu
615 N Wolfe St E8647
Baltimore, MD 21205
410-614-1355

Scan for LinkedIn profile:



EDUCATION:

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
PhD Candidate, 2010 - *Present*

University of Maryland, Baltimore County, Baltimore, MD
Bachelor of Science, Biological Sciences, 2006 – 2010

LABORATORY EXPERIENCE:

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2010 -
Present

Department of Biochemistry & Molecular Biology, Advisor: Dr. Anthony Leung
PhD Candidate

Project: Poly(ADP-ribose), or PAR, is a cellular polymer implicated in DNA/RNA metabolism, cell death, and cellular stress response via its role as a post-translational modification. The nature of these PAR polymers (large, charged, heterogeneous, base-labile) has made its amino acid attachment sites difficult to study by mass spectrometry. My thesis work has produced a pipeline which allows for the enrichment and identification of (ADP-ribosyl)ation sites via the enzymatic product of phosphodiesterase-treated ADP-ribose, phospho(ribose). My current focus is on characterizing the mono- and poly(ADP-ribosyl)ated proteome by application of this novel technique, the result of which would represent the first unbiased (by amino acid attachment-site) look into this important proteome.

University of Washington, Seattle, WA *Fall of 2012*

Department of Pharmacology, Advisor: Dr. Shao-En Ong
Visiting PhD Student

Project: To support my current thesis project (see above) I spent 7 weeks training in the field of proteomics under the mentorship of Dr. Shao-En Ong, an expert in phosphoproteomics and a pioneer in the field of SILAC.

University of Maryland, Baltimore County, Baltimore, MD 2009-2010

Department of Chemistry & Biochemistry, Mentor: Dr. James Fishbein
Research Assistant

Project: N-nitrosomorpholine (NMOR) is a potent carcinogen and mutagen, formed by the reaction of morpholine (a cheap, commercially available chemical) with sodium nitrite (found in the human body) in acidic media. Metabolism of NMOR occurs in the liver by P-450 enzymes and is consequently a proponent of liver cancer, along with esophageal and nasal tumors. My project centered around

studying the effects of NMOR intermediates on DNA, hypothesizing its spontaneous decomposition and further potential to form crosslinks via nucleoside adducts.

Wyeth (Pfizer) Pharmaceuticals, Collegeville, PA Summer of 2008

Department of Nuclear Receptors and Dermatology, Mentor: Dr. Catherine Thompson

Research Intern

Project: Explored the role of Liver X Receptor beta (LXR β) in mouse epidermis to determine whether LXR β is a therapeutic target for improving the integrity and function of aged skin. A member of the nuclear receptor superfamily, LXR β is a transcription factor that regulates specific genes in response to ligand binding. LXR β is highly expressed in the skin, and LXR ligands induce the expression of genes that are important for skin function, such as those that regulate lipid metabolism.

University of Maryland, School of Medicine, Baltimore, MD 2007 - 2008

Department of Pharmacology and Experimental Therapeutics, Advisor: Dr. Angelika Burger (dec.)

Research Assistant

Project: Tested the efficacy of the analogue 2'-F-ara-deoxyuridine (a cancer therapy drug currently in pre-clinical trials) as a suicide prodrug when used on cell lines from malignant human lung, breast and colon tumors which were grown in mice.

Siegfried Pharmaceuticals, Pennsville, NJ Summer of 2007

Department of Quality Control, Advisor: Paul V. Laraia, Jr.

Intern

ACADEMIC HONORS:

2014 - 2015	The Elsa Orent Keiles Fellowship in Biochemistry The Johns Hopkins Bloomberg School of Public Health
2006 – 2010	Academic Enhancement Award University of Maryland, Baltimore County
2007 & 2009	Semester Academic Honors University of Maryland, Baltimore County
2007	Dean's List University of Maryland, Baltimore County
2007	President's Award University of Maryland, Baltimore County

TEACHING EXPERIENCE:

- 2014 **Undergraduate Student Advisor**
Student: Lingga Adidharma
Contact: ladidharm@gmail.com, 1-307-460-8236
- 2014 **PhD Rotation Student Advisor**
Student: Pola Olczak
Contact: polaolczak7@gmail.com, 1-443-683-7059
- 2015 **PhD Rotation Student Advisor**
Student: Robert (Lyle) McPherson
Contact: rmchpher5@jhu.edu, 1-847-715-6811

RESEARCH GRANTS:

- | | |
|-------------------|---|
| 11/2014 – 5/2015 | Joy Cappel Young Investigator Award, Rockland
Immunochemicals
\$4000 |
| 4/2014 | Travel Award, Johns Hopkins Bloomberg School of Public Health
Student Assembly
Applied to “PARP Family & Friends” Conference
\$350 |
| 4/2014 | Travel Award, Cold Spring Harbor Laboratory
Applied to “PARP Family & Friends” Conference
\$100 |
| 7/2013 | Tuition Assistance, Cold Spring Harbor Laboratory
Applied to CSHL Proteomics Training Course
\$750 |
| 7/2011 – 6/2013 | Training Grant 5 T32-CA009110-35 (PI: Miller), National Cancer
Institute
Training in Areas Fundamental to Cancer Research
Full Stipend Support |
| 10/2012 – 12/2012 | Travelling Fellowship TF261, Journal of Cell Science
Supported training with Dr. Shao-En Ong in Seattle, WA
\$3,250 |

PUBLICATIONS:

“The Promise of Proteomics for the Study of ADP-ribosylation”

Casey M. Daniels, Shao-En Ong, Anthony K.L. Leung

Molecular Cell **2015** 58 (6), 911-924

PMID: 26091340

“Nudix hydrolases degrade protein conjugated ADP-ribose”

Casey M. Daniels, Puchong Thirawatananond, Shao-En Ong, Sandra B. Gabelli, Anthony K.L. Leung

Manuscript in preparation

“A phosphoproteomic approach to characterize mono- and poly(ADP-ribosyl)ation sites from cells”

Casey M. Daniels, Shao-En Ong, Anthony K.L. Leung

Journal of Proteome Research **2014** 13 (8), 3510-3522

PMID: 24920161

PRESENTATIONS & PUBLISHED ABSTRACTS:

2015 “Characterizing ADP-ribosylation sites by Mass Spectrometry” – poster

C.M. Daniels*, S.E. Ong, A.K.L. Leung

Annual Retreat – Department of Biochemistry & Molecular Biology

Johns Hopkins Bloomberg School of Public Health – Baltimore, MD

2014 “Using Mass Spectrometry to Identify Sites of ADP-ribosylation” – poster

C.M. Daniels*, S.E. Ong, A.K.L. Leung

PTMs in Cell Signaling – 6th Copenhagen Bioscience Conference, Denmark

2014 “A phosphoproteomic approach to characterize mono- and poly(ADP-ribosyl)ation sites from whole cell lysate” – poster

C.M. Daniels*, S.E. Ong, A.K.L. Leung

MaxQuant Summer School, National Institutes of Health, Bethesda, MD

2014 “A phosphoproteomic approach to characterize mono- and poly(ADP-ribosyl)ation sites from whole cell lysate” – poster

C.M. Daniels*, S.E. Ong, A.K.L. Leung

American Society for Mass Spectrometry (ASMS) Conference, Baltimore, MD

2014 “A phosphoproteomic approach to characterize mono- and poly(ADP-ribosyl)ation sites from whole cell lysate” – poster

C.M. Daniels*, S.E. Ong, A.K.L. Leung

The PARP Family & Friends: Gene Regulation and Beyond, Cold Spring Harbor, NY

2014 “Mass Spectrometry and ADP-ribosylation” – oral presentation

C.M. Daniels*, S.E. Ong, A.K.L. Leung

Annual Retreat – Department of Biochemistry & Molecular Biology
Johns Hopkins Bloomberg School of Public Health – Baltimore, MD

- 2013 “Applying Mass Spectrometry to the Study of Poly(ADP-ribosylation)” – oral presentation
C.M. Daniels*, S.E. Ong, A.K.L Leung
Annual Retreat – Department of Biochemistry & Molecular Biology
Johns Hopkins Bloomberg School of Public Health – Baltimore, MD
- 2012 “Using SILAC and Mass Spectrometry to identify targets of PARP-1” – poster
C.M. Daniels*, S.E. Ong, A.K.L Leung
Annual Retreat – Department of Biochemistry & Molecular Biology
Johns Hopkins Bloomberg School of Public Health – Baltimore, MD
- 2009 “How Skin Ages: Exploring the Role of the Nuclear Receptor LXR β in the Skin” – poster
C.M. Daniels*, Q. Shen, W. Wang, C.C. Thompson
Undergraduate Research and Creative Achievement Day
University of Maryland, Baltimore County – Baltimore, MD
- 2008 “The Role of Thymidine Kinase and Thymidylate Synthase in the Response of Tumor Cells to the Suicide Prodrug 2'-F-ara-Deoxyuridine” – poster
P. Phatak*, C.M. Daniels, A.F. Shields, J.M. Collins, P.M. LoRusso, A.M. Burger
EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics - Geneva, Switzerland
Abstract published in the **European Journal of Cancer**: October 2008, 6(12), p. 150
- 2008 “How Skin Ages: Exploring the Role of the Nuclear Receptor LXR β in the Skin” – poster
C.M. Daniels*, Q. Shen, W. Wang, C.C. Thompson
Mid-Atlantic Pharmacology Society's Annual Meeting - King of Prussia, PA
Abstract published in **The Pharmacologist**: December 2008, 50(4), pg 210
- 2008 “The Role of Thymidine Kinase and Thymidylate Synthase in the Response of Tumor Cells to 2'-F-ara-deoxyuridine” – poster
C.M. Daniels*, P. Shelton, P. Phatak, C. Burgess, K. Natharajan, P. LoRusso, A.M. Burger
UMBC's Undergraduate Research and Creative Achievement Day – Baltimore, MD

TRAINING COURSES:

MaxQuant Summer School, National Institutes of Health, Bethesda, MD
Proteomics Student, July 2014

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Proteomics Student, July 2013

REFERENCES:

Anthony K.L. Leung, PhD
Assistant Professor, Department of Biochemistry & Molecular Biology
Johns Hopkins Bloomberg School of Public Health
410-502-8939, anleung@jhsph.edu

Shao-En Ong, PhD
Assistant Professor, Department of Pharmacology
University of Washington
206-616-6962, shaoen@uw.edu

Pierre Coulombe, PhD
Chair, Department of Biochemistry & Molecular Biology
Johns Hopkins Bloomberg School of Public Health
410-955-3671, pcoulomb@jhsph.edu

LABORATORY SKILLS:

Molecular Cloning

Site Directed Mutagenesis
Agarose Gel Electrophoresis
PCR amplification

Protein Analysis

Bacterial & Mammalian Cell Culture (SILAC, iTRAQ)
Protein expression & purification
Microscopy
SDS Page and Western Blotting
Fast Protein Liquid Chromatography (FPLC)
Mass Spectrometry (MS1 and MS2)
Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC)

Synthetic Chemistry

Prep Plate and Column Chromatography
Recrystallization
Distillation
Extraction

Analytical Chemistry

Nuclear Magnetic Resonance (NMR, ^1H , ^{13}C)
High Pressure Liquid Chromatography (HPLC)
Thin Layer Chromatography (TLC)

Data analysis and presentation (General)

Adobe Illustrator and Photoshop (Proficient)
Microsoft Excel, Word and PowerPoint (Proficient)
GraphPad Prism
R

Data analysis (Molecular Biology)

MultiGauge (Proficient)
Image Studio (Proficient)
ApE (Proficient)
UNICORN (Proficient)
ImageJ (Proficient)

Data analysis (Mass Spec and Proteomics)

MaxQuant (Proficient)
Protein Prospector (Proficient)
Thermo Xcalibur (Proficient)
Scaffold
Mascot
PEAKS
Skyline
Perseus